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INTERACTION OF ALPHA- GAMMA-MSH ANALOGUES WITH MC1, MC3 AND MC4 MELANOCORTIN RECEPTORS

Submitted by Peijing Peng

for the degree of

Doctor of Philosophy

of the University of Bath

1997

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Dedication

This thesis is dedicated to my parents who have given me constant encouragement, love and care throughout years of study.

Acknowledgements

I would like to thank my supervisors Dr. Colin W. Pouton and Dr. Stephen H. Moss for their constant support and encouragement for this project. Special thanks to Dr. Ulrike G. Sahm for her kind and patient help and support throughout the studentship. I am indebted to Dr. Graham Smith for patiently proof-reading my English. I am grateful to Dr. Richard G. Kinsman for his knowledgeable advice on peptide synthesis. I would like to thank Mr. Hatem A. Hejaz for his advise with computer. I would also like to thank the University of Bath for providing research facilities and the technical staff in the department of Pharmacy and Pharmacology for their assistance.

There are many other good friends I would like to thanks for their constant encouragement, and sharing ideas to make the life here more pleasant: Paul, Mrs. Keating, and Hazel.

Above all, many thanks to my parents for everything.

Abbreviations

ACTH	adrenocorticotropic hormone
BSA	bovine serum albumin
cAMP	cyclic adenosine 3'-5'monophosphate
Ci	Curie
CLIPCorticotrop	Corticotrophin-like intermediate lobe peptide
CNS	Central nervous system
cpm	counts per minute
DAG	diacylglyceride
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DMF	dimethylformamide
dpm	decays per minute
EDTA	ethylenediaminetetraacetic acid
EC_{50}	concentration of ligand required for 50% maximal response
FAB-MS	fast atom bombardment mass spectroscopy
FBS	foetal bovine serum
Fomc	Fluorenylmethocycarbonyl
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HEK	human embryonic kidney (cells)
HEPES	N-(2-hydroxyethyl)piperazine-N-2-ethane sulphonic acid
hMC-R	human melanocortin receptor
HPLC	high pressure liquid chromatography
IBMX	1-methyl-3-isobutylxanthine
IC_{50}	concentration of ligand required to inhibit 50% maximal response
IL	interleukin
IP_3	1,4,5,-triphosphate
K_d	dissociation constant of radioligand
K_i	dissociation constant of competitor

LPH	lipotropin
μ	micro (10^{-6})
M	mole per liter
MC	melanocortin
mMC-R	murine melanocortin receptor
mRNA	messenger ribonucleic acid
MSH	melanocyte-stimulating hormone
n	nano (10^{-9})
NMR	nuclear magnetic resonance
NO	nitric oxide
PBS	phosphate buffered saline
PCA	perchloric acid
PDE	phosphodiesterase
PKA	protein kinase A
PKC	protein kinase C
POMC	pro-opiomelancortin
RER	rough endoplasmic reticulum
TFA	trifluoroacetic acid
TNF	tumor necrosis factor
Tris	tris (hydroxymethyl) aminomethane
TRP	tyrosinase-related protein
UV	ultraviolet
v/v	volume by volume
w/v	weight by volume

CONTENTS

Abstract

Five classes of melanocortin receptors (MC1, MC2, MC3, MC4, and MC5) have been cloned to date. The MC1-R was found in melanocytes and melanoma cells, and macrophages, and shows high affinity and activity for α-MSH, while MC2-R is an ACTH receptor expressed in the adrenal gland. The MC3-R, MC4-R, and MC5-R have been found in brain and peripheral tissue, however, the function of the MC3-R and MC5-R remain unknown. It has been reported that mouse agouti peptide displays antagonist activity at MC4-R, and there are indications that the MC4-R is involved in the control of feeding behaviour.

The significance of the structure-activity relationship are discussed in the context of current knowledge.

The work presented in this thesis focus on the relationship between structure and activity of α-MSH and γ-MSH analogues at the mouse MC1-R, rat MC3-R and rat MC4-R. In this study the native MC1-R was expressed by B16 murine melanoma cells, and cDNAs encoding the rat MC3-R and human MC4-R were stably expressed by transformed human embryo kidney cells (HEK 293). Initially substitution was carried out at position 12 of α-MSH to test the importance of this position in interaction of α-MSH and γ-MSH with the MC1-R and MC3-R. In later studies modification of [Phe^7] was investigated at the MC1-R, MC3-R, and MC4-R in an effect to find a selective and potent ligand for each receptor. Cyclic peptides of [Nle^4,$\overline{Asp^5,D\text{-}Phe^7,Lys^{10}}$]α-$MSH_{4\text{-}13}$ were studied at the MC1-R to investigate the properties of these bioactive conformers which have less flexibility and may be constrained in a 'receptor-specific' conformation. Throughout the study α-MSH analogues were synthesised by solid-phase techniques and assayed for both receptor binding (by competitive inhibition) and stimulation (as determined by cAMP production).

Chapter 1: INTRODUCTION

1.1. Melanocytes and Melanoma

The skin protects the body against heat, light, infection, and injury. It also stores water, fat, and vitamin D. The skin consists of two main parts, the epidermis and dermis, and several kinds of cells. The outer layer of skin is called the epidermis and the inner layer dermis. The Epidermis contains 4 layers[1] (Fig.1.1). 1) The basal cell layer (stratum basale) comprised mostly of keratinocytes, and melanocytes, which are able to synthesise melanin. 2) The prickle cell layer (stratum spinosum), which consists of basal cells that migrate upwards to form a layer of polyhedral cells. 3) The granular layer (stratum granulosum), contains cells which become flattened and lose their nuclei. 4) The horny layer (stratum corneum) is the end result of keratinocyte maturation, and is comprised of sheets of overlapping polyhedral cells with no nuclei (corneocytes).

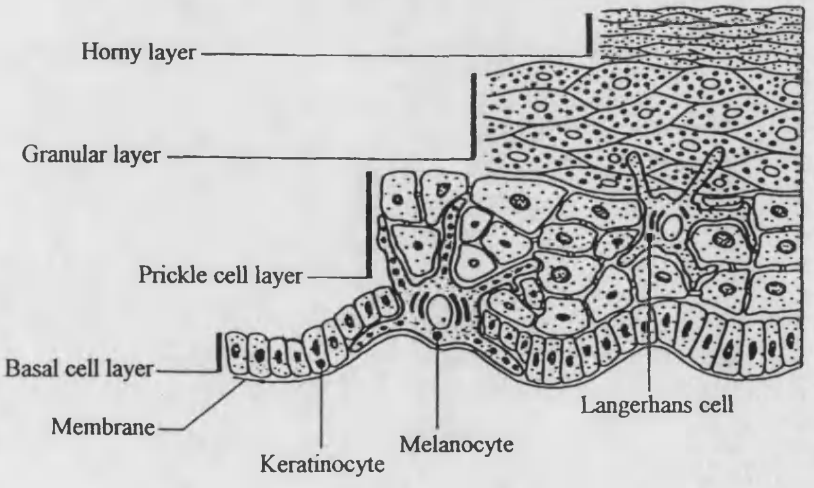

Figure 1.1: Layers of the epidermis and other structures[1].

The fashionable demand for tanned skin, and loss of the protective ozone layer because of environmental pollution (choroflurocarbon, methane, etc.) have led to a dramatic increase in the number of people suffering from skin cancer. There are several types of cancer that start in the skin. The most common are basal cell and squamous cell cancers, which arise from the basal keratinocytes of the epidermis. The main difference between basal cell and squamous cell cancers is that basal cell cancer does, in general, not metastasise, whereas squamous cell cancer may metastasise. Malignant melanoma is a malignant tumour of melanocytes, usually arising in the epidermis and is the most lethal of the main skin tumours.

According to recent reports[2,3], the incidence of malignant melanoma has increased by 4 percent each year since 1973. About 38,000 cases of melanoma were diagnosed in the US in 1996 and approximately 7,000 deaths are expected[3]. Basal cell and squamous cell skin cancers, which are not as serious as melanomas[2] account for more than 800,000 skin cancer cases in the US annually but cause only 2,100 deaths because they are highly curable.

1.2. Treatment of Skin Cancer

There are several approaches to treatment of skin cancer, depending upon the particular type, but the preferred treatment remains surgery[4]. Surgery, however, may not only scar the skin, depending on the size of the cancer, but complete removal of the tumour is not always successful for malignant melanoma, which has a tendency to metastasise at an early stage. Radiation therapy uses x-rays to eradicate cancer cells and shrink tumours for basal cell and squamous cell cancers and chemotherapy drugs are administered to kill cancer cells.

At present there are a number of both physical methods and new chemotherapeutic agents undergoing assessment for the management of advanced cutaneous malignancy. In general, it is unlikely that these approaches will result in the complete cure of advanced disease[4]. Recently, interferon and interleukin-2 are under clinical trial in melanoma. Interferon showed a 15 percent response rate in approximately 309 melanoma patients[5]. However, there were a number of detrimental side-effects such as fever; low blood count; and liver toxicity[4]. Interleukin-2 stimulates growth of T-cells *in vitro*, and *in vivo* also appears to activate killer cells. The report proposed a 50 percent response rate in malignant melanoma raised great hope[6]for it application as a clinical tool. But the significant toxicity includes dramatic fluid shift problems[4].

Any preferred treatment would be more specific with fewer side effects. One such approach is the use of MSH peptides. α-MSH is active in skin pigmentation, and melanocortin receptor MC1-R is specific for α-MSH, so, study of the relationship between α-MSH and MC1-R may have potential as a new approach to therapy or as an aid in the diagnosis of skin cancer.

Peptide hormones such as α-MSH are key regulators many of cellular, intercellular, organ, and other physiological processes, so they may not be as toxic as current drugs[7]. α-MSH has a number of attractive features as a model peptide. As well as being a natural hormone, α-MSH is 13 amino acids which is large enough to provide technically the opportunity for significant structural alteration in the preparation of analogues and is small enough to facilitate synthetic procedures. There are many reports in the literature about the physiological roles of α-MSH *in vivo* and a number of melanoma cell lines are known to express α-MSH receptors[8-

[11]. This will be discussed in more detail later in this chapter. Recently, molecular biology techniques have provided a combination of specific and comprehensive *in vitro* and *in vivo* bioassays and binding assays to provide detailed descriptions of the biochemical and biophysical events related to biological activity. The characterisation of partial agonists or antagonists may lead to the development of receptor antagonists as potential therapeutic agents. It is important to recognise that most peptide hormones interact with complex multiple receptor subtypes, and it is necessary to use multiple assays to obtain insights into the differential structural, conformational and dynamic requirements for each individual receptor subtype. Thus, studying the different receptors in order to define different physiological roles of MSH is another approach to the design of potential therapeutic agents. Several attempts have already been unable to exploit α-MSH in specific during targeting to melanoma.

α-MSH and β-MSH[12-15] have been conjugated to FAB fragments of antibodies for drug targeting. Their uptake into cells is receptor-mediated, and specific for certain cell types. However, most of them have demonstrated poor results when carried out *in vivo* for most receptors are widely distributed in different tissues, making selective delivery impossible by MSH-antibody conjugates. α-MSH analogues have also been tested clinically for other purposes.

Two compounds are in clinical studies as potential tanning agents without the need for sunlight exposure. [Nle^4,D-Phe^7]α-MSH called Melanotan-I (MT-I) has shown to be non-toxic in mice[16]. A preliminary clinical trial of MT-I in normal males showed that tanning could be achieved in the absence of sunlight exposure, and

there were only minor side effects, consisting of transient facial flushing and rarely, gastrointestinal upset[17].

Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}] α-MSH$_{4-10}$, called Melanotan-II (MT-II), is able to increase human skin pigmentation at much lower dose than MT-I. MT-II, however, has more potent dose-related side effects such as mild nausea. At lower dose, MT-II interacts with MC1-R in melanocytes to produce tanning, and at high doses, with MC3-R in the brain to produce fatigue and nausea[18].

1.3. POMC Peptides

Hormones are chemical messengers manufactured by the endocrine glands. They regulate the function of many vital organs. Hormones fall into four categories[19] : 1) Peptide, secreted by the anterior pituitary. 2) Steroids, from the adrenal cortex and gonads. 3) Iodothyronines, from the thyroid gland. 4) Catecholamines, from adrenal medulla. There are differences in the synthesis, physical state in the circulation, half life, metabolism, and mechanism of action of these four hormonal groups.

Peptide hormones are synthesised from large precursors and stored in the gland of origin. They are soluble in aqueous solvents, circulate in unbound form and have a half-life of minutes. Peptide hormones may act at the plasma membrane by binding to specific receptors and stimulating the synthesis of cyclic adenosine monophosphate (c-AMP) and other second messengers (as described in section 1.3.1.).

Steroid hormones are synthesised by a series of enzymatic reactions from a common precursor, cholesterol. Glandular storage of steroids is minimal. They are

soluble in nonaqueous solvents, circulate predominantly bound to plasma proteins, and have a half-life of hours. Steroids cross the plasma membrane of target cells, bind to cytoplasmic receptors, and act at the nucleus by initiating transcription.

Iodothyronines are similar to steroid hormones in many respects. They are synthesised in the thyroid gland from iodine and tyrosine. The half-life of iodothyronines in circulation is longer than that of steroids (a span of days).

Catecholamines share many of the properties of peptide hormones, but their synthesis, like that of steroids and iodothyronines, involves a series of enzymatic modifications of a precursor, in this case phenylalanine.

1.3.1. Biosynthesis of Peptide Hormones

Peptide hormones are synthesised in the rough endoplamic reticulum, translocated to the Golgi, where they are modified (glycosylated, phosphorylated, acetylated, amidated, sulphated or methylated) in order to be secreted[20,21]. Secretion of peptide hormones occurs by exocytosis, where the storage granule is translocated to the cell surface and its membrane joins with the plasma membrane. Peptide hormones are delivered to specific target cells where they are recognised by specific receptors. Peptide hormones interact reversibly with receptors on the surface of the target cell. Intermediate molecules called second messengers deliver the signal to the intracellular organelles responsible for carrying out the hormonal command. c-AMP is the second messenger for many peptide hormones including α-MSH (Fig.1.2).

c-AMP is formed from ATP within the cell by the action of adenylate cyclase, which is located on the inner face of the plasma membrane. Adenylate cyclase is

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the catalytic part of the hormone-receptor complex, which consists of at least two other components: the recognition component, which binds to the hormone, and a regulatory protein, which couples the recognition component to the catalytic portion. The regulatory protein, which binds guanine nucleotides, is called the G protein or N protein. Binding of hormone by the recognition component prompts the regulatory subunit to replace its guanosine diphosphate (GDP) with guanosine triphosphate (GTP), activating the catalytic portion. While the catalytic unit is resting, the inactive state is achieved by hydrolysis of GTP to GDP via the regulatory protein. The presence of GTP on the regulatory protein lowers the affinity of the recognition component for the hormone, and the bound hormone is released. The inhibitory actions involve a guanine nucleotide binding regulatory protein, which inhibits the adenylate cyclase component. Because the guanine nucleotide regulatory proteins may be either stimulatory or inhibitory they are called Gs and Gi.

c-AMP produces its physiological effect by activating protein kinase A (PKA). PKA has regulatory and catalytic subunits; c-AMP binds reversibly to the regulatory subunit, causing it to dissociate from the catalytic subunit, which is then free to exert its physiological activity (Fig.1.3). c-AMP is destroyed by cyclic AMP phosphodiesterase (PDE), which converts it to 5`-AMP. The decrease of cAMP allows the re-association of regulatory and catalytic subunits of PKA, thus it inactivates.

Calcium has been recognised as a major regulator of cellular processes. It plays an important role in activation of various enzymes. The concentration of free calcium in the cytoplasm of resting cells is low, about 1 micromolar or less. Upon activation of the cell by neural or hormonal signals, the free calcium concentration

rises promptly, increasing perhaps 1000-fold and may reach the millimolar range. This is accomplished for most cells by a release of calcium from intracellular

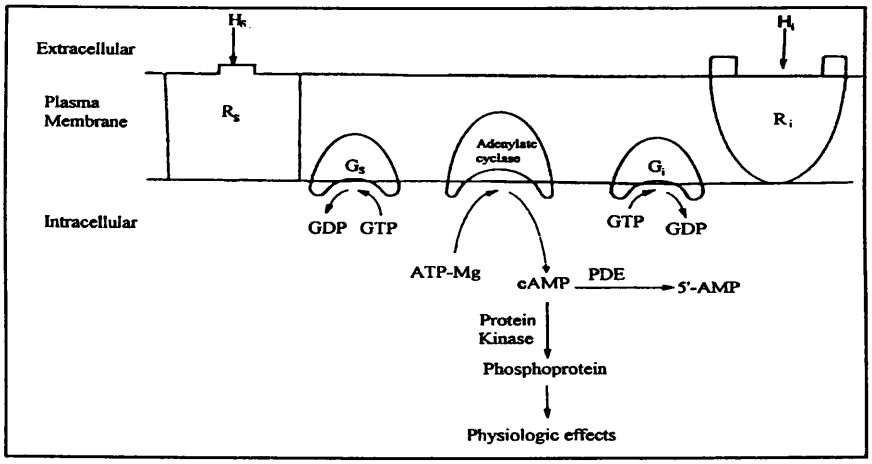


Figure.1.2.: Adenylate cyclase system. Hs= stimulatory hormone. Hi= inhibitory hormone Rs and Ri= recognition components of the receptor complex. Gs and Gi= guanosine nucleotide binding proteins. PDE= cyclic nucleotide phosphodiesterase, which inactivates cAMP. The plot is base on[20].

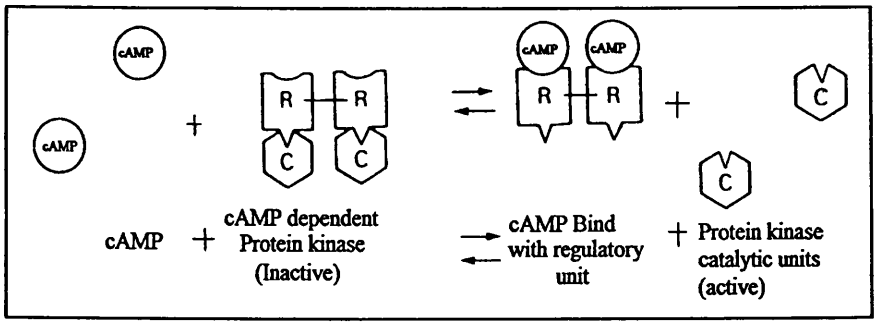


Figure.1.3: Activation of protein kinase A by cAMP. Inactive protein kinase consists of catalytic units (C), which binds with regulatory unit (R). When cAMP binds to the regulatory unit, the catalytic unit is released and activated. A decrease in cAMP allows the regulatory unit to rebind to the catalytic unit. The plot is adapted from[20].

storage sites mainly within the endoplasmic reticulum and by influx of calcium from the extracellular fluid. Virtually all cells are endowed with a protein called calmodulin, which is the intracellular receptor for calcium. When bound to

calcium, calmodulin is able to bind to a variety of cellular proteins and activate them. When some hormones or neurotransmitters bind to their receptors on the surface of cells, they activate an enzyme, phospholipase C, which splits the membrane phospholipids, phosphatidylinositol 4,5-bisphosphate, into diacylglyceride (DAG) and inositol 1,4,5-triphosphate (IP_3). Both of these compounds behave as second messengers. The role of IP_3 is to mobilise intracellular calcium from storage sites in the endoplasmic reticulum, and DAG activates protein kinase C (PKC), which catalyses the phosphorylation of various cellular proteins to activate or inhibit them[20].

The mechanism of action of α-MSH and related peptides have been reported. In a cAMP assay, melanoma cells showed a dose-dependent cAMP increase on treatment with α-MSH, [Nle^4,D-Phe^7]α-MSH, and β-MSH[22,23]. Freshly isolated intact E15 rat Dorsal Root Ganglia (DRG) showed a dose-response curve with a maximal increase in cAMP production of 70-85%, when treated with 1-10 nM α-MSH or 10 nM ORG2766[24]. With regard to intracellular calcium/inositol phosphate responses, there are indications that phosphoinositides play a role in the mechanism of action of melanocortins in CNS tissue. Lysed synaptosomes treated with 100 μM $ACTH_{1-24}$, $ACTH_{1-16}$, or α-MSH show an increase in IP_3[25]. In the absence of the melanotropin, frog (*Rana pipiens*) skins previously darkened with [Nle^4,D-Phe^7]α-MSH, a potent synthetic analogue of α-MSH, could be lightened by removal of calcium from the incubation medium but could be redarkened by adding calcium[26]. Thus, MSH darkening actions increase in the presence of calcium[27].

α-MSH enhances PKC in B16 mouse melanoma cells[28]. Some reports have shown that PKC is involved in human[29] and murine melanogenesis[30] by activating tyrosinase. Depletion of PKC results in a complete inhibition of α-MSH induced melanogenesis[30].

It is possible that some receptors are linked to the formation of more than one second messenger or that different classes of receptor for a single hormone exist on a single cell, each with its own second messenger[19]. For example, Konda *et al.*[31] demonstrated that at the MC3-R both α-MSH and ACTH at human were able to stimulate both cAMP/PKA and inositol phospholipid/Ca^{+2} at concentrations as low as 10^{-11} M.

1.3.2. Peptides Encoded by the Pro-opiomelanocortin Gene

Studies of adrenocorticotropic hormone (ACTH) led to the discovery of pro-opiomelanocortin (POMC). When Mains *et al.*[32] and Roberts and Herert[33,34] demonstrated that a larger protein contained both ACTH and β-endorphin, the precursor, POMC, was soon identified[35]. Nakanishi *et al.*[35] determined the amino acid structure of bovine POMC, providing for the first time the complete sequence of the molecule. They used the nucleotide sequence of cloned DNA complementary to POMC mRNA to predict the entire amino acid sequence of the protein. POMC was found to be released from pituitary gland, and cleaved into three parts: The N-Terminal peptide (103 amino acids), ACTH (39 amino acids), and β-lipotropin (β-LPH, 93 amino acids). The N-terminal peptide is processed to yield γ-MSH, while ACTH is processed in some tissues to α-MSH and

corticotropin-like intermediate lobe peptide (CLIP), and β-LPH gives rise to γ-LPH, β-MSH, and β-endorphin (Fig.1.4)[21].

Studies of the mechanisms involved in the production of bioactive peptides from POMC show that different peptides are produced from POMC in different tissues. This introduced the concept of tissue specificity to the processing of peptide hormone[21].

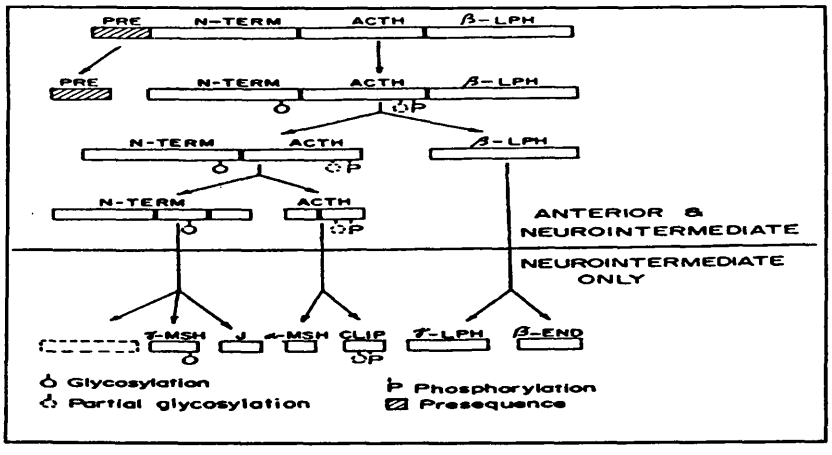


Figure 1.4: Tissue-specific processing in the two lobes of the rat pituitary. In the anterior lobe POMC is only processed to yield the N-terminal peptide, ACTH, β-LPH, whereas in the neurointermediate lobe POMC is further processed to yield γ-MSH, the joining peptide, α-MSH, CLIP, γ-MSH, and β-endorphin[21].

1.3.3. Tissue-specific Processing of POMC

The anterior and neurointermediate lobes of the rat pituitary provide means of studying tissue-specific expression of POMC-derived peptides. The anterior lobe contains predominantly $ACTH_{1-39}$, as where in the neurointermediate lobe $ACTH_{1-39}$ is processed to yield α-MSH ($ACTH_{1-13}$ with an acetylated N-terminal and amidated C-terminal) and CLIP ($ACTH_{18-39}$)[36]. The two lobes also differ in the amounts of β-LPH and β-endorphin produced[37].

1.3.3.1. α-MSH

Mammalian α-MSH (Ac-Ser[1]-Tyr[2]-Ser[3]-Met[4]-Glu[5]-His[6]-Phe[7]-Arg[8]-Trp[9]-Gly[10]-Lys[11]-Pro[12]-Val[13]-NH_2) is a basic tridecapeptide. It also exists in des-acetyl- or di-acetyl forms. Its amino acid sequence was first published in 1957 by Harris and Lerner[38]. α-MSH is not only found in the brain but also in peripheral tissues, e.g. skin, gastrointestinal tract, placenta[39]. α-MSH release from the CNS is controlled by MSH releasing factors (MRF) and inhibiting factors (MIF). Some mammalian MIFs are dopamine, γ-aminobutyric acid (GABA), enkephalin, melatonin, serotonin, somatostatin, and adenosine[40]. Some MRFs are (nor)adrenaline, vasopressin, corticotrophin releasing factor (CRF) and opiates[41,42].

1.3.3.1.1. Distribution

In most vertebrates, α-MSH is formed mainly in the pars intermediate of the pituitary gland. Adult humans lack a pars intermedia, but it is present in the human foetus. Thus, the human foetus contains a high α-MSH bioactivity which decreases after birth[43,44]. Rudman *et al.*[45,46] studied the occurrence and localisation of MSH peptides in various regions of bovine, simian, human and rat brain. The highest concentration of MSH in the brain is found in the hypothalamus[47].

α-MSH bioactivity or immunoreactivity has been found in various peripheral tissues, such as skin[48], testes[49], placenta[50], and gastrointestinal tract[51]. Plasma α-MSH levels have been reported as being elevated in some melanoma patients with

an increase of about one third above levels found in controls[52]. A more detailed review of the biological actions of α-MSH is given in section 1.4.

1.3.3.2. β-MSH

The C-terminal of POMC cleavage fragment β-lipotropin (β-LPH), which is processed further to γ-lipotropin (γ-LPH), β-endorphin and β-MSH. β-MSH is an octadecapeptide and corresponds with the sequence γ-LPH_{41-58}. The structure of β-MSH in different vertebrates is more variable than that of α-MSH. Certain species produce two forms of β-MSH, indicating either the expression of two different genes or a posttranslational modification. In common with α-MSH, β-MSH contains the heptapeptide sequence: Met-Glu-His-Phe-Arg-Trp-Gly.

1.3.3.3. γ-MSH

γ-MSH is this $ACTH_{4-10}$-like sequence displaying structural homology to be α-and β-MSH peptides potentially derived from it. Although Nakanishi *et al.*[35] only hypothesised the natural occurrence of a 12 amino acid residue "γ-MSH", Ling *et al.*[53] predicted the existence of a family of γ-MSH peptides, using the presence of basic amino acid pairs and established putative posttranslational processing reactions; e.g., NH_2-terminal acetylation or COOH-terminal glycine deletion and amidation. Three classes of γ-MSH peptides were synthesised accordingly: γ_1-MSH, containing 11 amino acid residues with an amidated COOH-terminal Phe; γ_2-MSH, a COOH-terminal Gly extension of γ_1-MSH; and γ_3-MSH, a 15-amino

acid residue extension of γ_2-MSH[52]. The majority of the naturally occurring γ-MSH peptides contains a lysine residue in the N-terminal region[39]. The structures of γ-MSH are as follows:

γ_1-MSH H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2

γ_2-MSH H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH

γ_3-MSH H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Arg-Arg-Asn-Gly-Ser-Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln-OH

[Lys]γ_1-MSH H-Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2

[Lys]γ_2-MSH H-Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH

Shibasaki *et al.*[54] have demonstrated that only γ_1-MSH immunoreactivity could be found in bovine neurointermediate lobe, while γ_3-MSH was the product of NH_2-terminal POMC posttranslational processing in bovine anterior lobe. γ_1-and γ_2-MSH are the predominant molecular species present in human pituitary[55]. Fodor *et al.*[56] demonstrated that [Lys]γ_2-MSH and γ_2-MSH are widespread in rat brain, where they are found in intermediate and anterior lobes of the pituitary gland, the hypothalamic arcuate nucleus and the commissural part of the nucleus of the solitary tract (NTS). The occurrence of [Lys]γ_2-MSH immunoreactivity in many of the brain regions which are involved in cardiovascular regulation suggests a role of γ-MSH in the regulation of cardiac function; The iv. administration of γ_2-MSH and [Lys]γ_2-MSH in rats produced dose-dependent increases in arterial pressure and significant cardioacceleration[57,58]. Similar effects were noted for γ_1-MSH[59], but γ_3-MSH had no presser activity[58].

Recently, Bergen *et al.*[57] showed that C-terminal shortening of γ_2-MSH resulted in γ-MSH peptides which had no effect on mean arterial pressure and heart rate.

These results suggest that the intrinsic activity of γ_2-MSH is carried by the C-terminal γ-MSH fragment Asp^9-Arg^{10}-Phe^{11}. It has been postulated that the cardiovascular effects of γ-MSH are dependent on the Arg-hydrophobic amino acid sequence located at or near its C-terminus[58]. α-MSH, γ_3-MSH and ORG2766, which do not contain this sequence in the C-terminus, show no cardiovascular activity in conscious rats[58,60,61].

Within the anterior pituitary, γ-MSH peptides were unable to evoke the release of luteinising hormone, follicle stimulating hormone, prolactin, growth hormone or thyrotropin[53]. Unlike γ-MSH, α-MSH was able to affect the release of other pituitary hormones via feedback loops. For example: α-MSH stimulated growth hormone and thyrotropin, inhibited prolactin release and modulated luteinising hormone[39]. γ-MSH peptides may not act as releasing factors for anterior pituitary hormones, thus they may exert other physiological effects.

γ_1-MSH, Ac-γ_1-MSH, γ_2-MSH and γ_3-MSH were tested in the *Rana pipiens* melanophore assay[53]. All peptides were able to stimulate melanocytes, but only at very high concentrations compared to α-MSH. The most active peptide in this assay was Ac-γ_1-MSH, a peptide synthesised in analogy to the N-terminus of α-MSH. γ_3-MSH was the least active peptide in the study.

So far, the specific receptors for γ-MSH in involved in mediating its cardiovascular action are unidentified, though, it has been postulated that some of the actions of γ-MSH are mediated by the MC3-R[62,63].

1.3.4. Biological Activity of Peptides Derived from POMC

Peptides derived from POMC have diverse activities in both the nervous system and peripheral tissues[64]. POMC derived peptides are thought to be involved in enhancing attention, behaviour, learning and memory[65,66], control of cardiovascular function[60], thermoregulation[67], analgesia[68], and effects on the immune system[69].

While the most important peripheral effect of α-MSH is stimulation of melanogenesis by pigment cells[39], ACTH is best known for regulating adrenal cortical cells and aldosterone production, and β-endorphin is for its analgesic effects[70,71]. Recently, γ_2-melanocyte stimulating hormone (γ_2-MSH) has been suggested to enhance effects on cerebral blood flow in the rat[61,72] . The melanogenic effects of ACTH/α-MSH related peptides were a suitable focus for this study, as it provided a means of investigating melanoma.

1.4. Biological Effects of α-MSH

1.4.1. Effect on Pigmentation

The most clearly defined physiological role of α-MSH is to control melanin pigmentation of the skin in most vertebrate species. In mammals and many other vertebrates melanocytes, melanin-producing pigment cells, are found in the basal layers of the epidermis. Melanocytes are derived from the neural crest from where they migrate as undifferentiated melanoblasts into the dermis and later invade the

epidermis and there differentiate into melanocytes[39]. These melanocytes in mammals (melanophores in lower vertebrates) utilise tyrosine to produce, through a number of biochemical events controlled by one or more enzymes, coloured polymers called melanins. Brown and black melanins are referred to as eumelanins, whereas red or yellow melanins are known as phaeomelanin (Fig.1.5). The biosyntheses of these compounds initially have a common metabolic pathway[73,74]. Tyrosine is hydroxylated to dihydroxyphenylalanine (DOPA) in eumelanin formation which is further oxidised to dopaquinone. Both steps are catalysed by tyrosinase, the enzyme in melanin biosynthesis. In eumelanin formation, Dopaquinone undergoes a spontaneous irreversible and rapid intramolecular cyclization reaction to form leucodopachrome which is further oxidised to dopachrome, then rearranges to 5,6-dihydroxyindole, is oxidised to indole-5,6-quinone and converted into melanochrome. For phaeomelanin biosynthesis, Dopaquinone proceeds through glutathionedopas, cysteinyldopas, cysteinyldopaquinones and 1,4-dihydrobenzothiazines, then phaeomelanin. At low tyrosinase activity, levels of dopa are low and the predominating pathway is the synthesis of cysteinyl-dopa which is incorporated into melanin to give phaeomelanin. On excess production of DOPA, as results after maximal stimulation by MSH, DOPA is converted to dopachrome which gives black eumelanin.

Mammals with varied coloured pelage pattern produce one or another of these melanins in different anatomical area of the skin[39]. α-MSH enhances melanin production within melanocytes, and the melanosomes produced migrate into the dendrite processes of the cells where they are released into the surrounding cells of

the epidermis. Repeated injections of α-MSH or a synthetic analogue of α-MSH into individuals increases epidermal pigmentation, which is readily visible after a few days, demonstrating the capability of the human melanocyte system to respond to α-MSH[75,76]. Recently it has been also shown that [Nle^4,D-Phe^7]α-MSH increased skin darkening in human by after injection[8].

Hunt *et al.*[77] showed that ACTH stimulated melanogenesis in cultured human melanocytes. Later, they also demonstrated that α-MSH increased tyrosinase activity and melanin content[78], especially eumelanogenesis[79] in humans epidermal melanocytes.

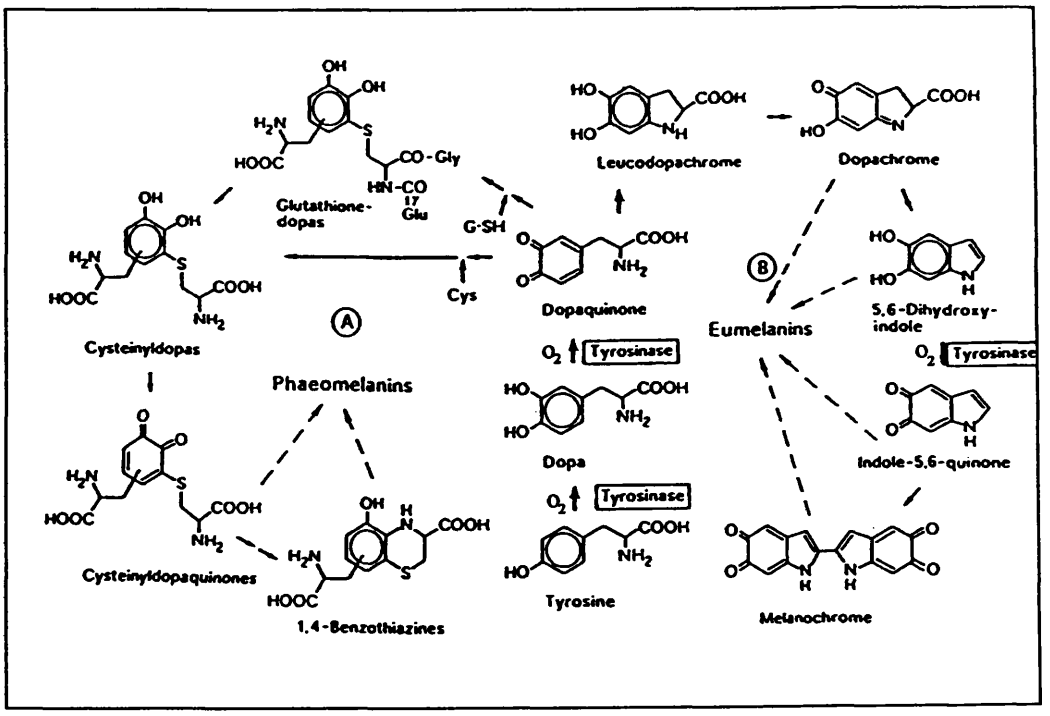


Figure.1.5: The melanin pathway[39]

1.4.1.1. Extension and Agouti

Melanogenesis in mammalian melanocytes leads to the formation of either eumelanin or phaeomelanin, resulting in a brownish and black or yellowish and

red coat colour, respectively. The pathways are controlled by the products of two classical coat-colour controlling gene loci, extension and agouti.

1.4.1.2. Extension

Extension encodes the melanocortin receptor MC1-R, which is G coupled protein. MC1-R binds α-MSH and is expressed on pigment cells. The extension locus extends the range of brown/black pigment when dominant, and blocks eumelanin synthesis when recessive, thereby extending the range of red/yellow pigment. Coat colours in wild and domesticated animals are often determined by extension alleles at what is thought to be a homologous gene locus[80].

1.4.1.3. Agouti

The murine agouti gene encodes for a novel 131 amino acid agouti protein. The sequence includes a secretion signal, a lysine-rich basic region, and a C-terminal domain containing 10 cysteines with the potential to form 5 disulfide bonds[81]. The importance of the signal peptide and the C-terminal cysteine residues for full biological activity have been established *in vitro*. But the lysine-rich basic region appears not to be essential for normal function[82].

The function of the agouti protein is to regulate the differential production of melanin pigments by melanocytes[83], by acting in a paracrine manner to shift melanogenesis towards phaeomelanin[84,85]. It competes with α-MSH in binding to MC1-R and inhibiting MSH-induced cAMP formation[81,86-88]. Siegrist *et al.*[87] proposed that the antiproliferative action of the agouti protein in B16 melanoma

cells *in vitro* could be caused by a down-regulation of MC1-R, resulting in lower levels of intracellular second messengers, such as cAMP, which are required for normal cell growth and function.

Agouti is also found to be an antagonist of MC4-R[86,89], but not MC3-R or MC5-R. When agouti is overexpressed in yellow mutant mice, it induces obesity, insulin resistance and increased susceptibility to neoplastic lesions[90,91]. Intracerebroventricular administration of the agouti-mimetic peptide SHU9119, a potent MC4-R antagonist, induced a significant increase in food intake[89].

Recent evidence has suggested that agouti might also increase intracellular Ca^{2+} concentrations via a mechanism that may not involve antagonism of the melanocortin receptor[92]. The agouti protein antagonises the effect of verapamil, a Ca^{2+} antagonist; on melanogenesis, suggesting that Ca^{2+}-dependent mechanisms may be involved in mediating the action of agouti protein[93].

1.4.1.4. UV Radiation

Melanin production in melanocytes can also be enhanced by factors other than α-MSH, such as UV radiation, especially UVB, which has been speculated to be a risk factor for the development and progression of human cutaneous melanoma[71,94]. Interactions between MSH, IL-1 and UV light were examined in Cloudman mouse melanoma and human squamous carcinoma cell lines[95]. The data suggested that both cell lines produced IL-1 and that production was stimulated by exposure of the cells to UV; both cell lines possessed high affinity binding sites for MSH, and their ability to bind MSH was modulated by IL-1; the stimulatory effect of IL-1 on MSH binding to melanoma cells was reflected in enhanced cellular

responsiveness to MSH regarding tyrosinase activity and melanin content. Recently, it has demonstrated that UVB radiation stimulates increased expression of the POMC gene, accompanied by production and release of MSH and ACTH by both normal and malignant human melanocytes and keratinocytes[96-98], where MSH receptors were found to be expressed[94]. Thus, UVB, and α-MSH, enhance MSH receptor activity in mouse melanoma cells[95,99] or human melanocytes[97,98].

1.4.1.5. Variants of the MC1 Receptor

Eumelanin is photoprotective whereas phaeomelanin may contribute to UV-induced skin damage. Individuals with red hair have a predominance of phaeomelanin in hair and skin or a reduced ability to produce eumelanin, which may explain why they fail to tan and are at risk from ultraviolet radiation(UVR)[100]. It has been reported that red hair and pale skin show strong association with variants of MC1-R[100], suggesting that the majority of changes found were at codon 92 and 294. Hunt *et al.*[101] extended the study and reported that unresponsiveness to MSH is particularly prevalent in epidermal melanocytes from individuals with red hair. It indicated that the lack of functional MSH receptors on these melanocytes among these groups was a factor in their failure to response to MSH[101]. Recently, it has also been reported that the Asp84Glu ,replacing Aspartate with Glutamate, variant was only present in melanoma cases and appears to be of particular significance[102].

1.4.2. Effect of α-MSH on the CNS

1) Effect on grooming and stretch-yawn behaviour

Intraventricular administration of many α-MSH and ACTH-like peptides in the nanogram to microgram range produces a series of vigorous stretching and yawning in rats[103-106]. α-MSH was the most potent in this assay, then β-MSH and $ACTH_{1-24}$, whereas γ_2-MSH, $ACTH_{4-10}$, and [Leu^9]α-MSH were inactive. The synthetic, "superpotent" peptide, [Nle^4,D-Phe^7]α-MSH had more activity than α-MSH[107].

2) Effect on sexual behaviour

α-MSH, ACTH, and related peptides have been reported to influence sexual behaviour in male and female rats. Both α-MSH and $ACTH_{4-10}$ are effective in inducing sexual behaviour in ovariectomized rats receiving both estrogen and progesterone[108,109].

3) Effects on arousal, attention, learning, and memory

The effects of $ACTH_{4-10}$ peptides have been investigated in human volunteers and patients, and results are consistent with an improvement in subject attention caused by these peptides. α-MSH and $ACTH_{4-10}$ have been reported to cause subjects to maintain high vigilance levels, prevent the decay of performance due to decreased

motivation and mental fatigue and improve visual attention and visual motor learning. Verbal memory, however, was not enhanced[110-112].

4) Effect on neural regeneration in the peripheral nervous system

Several authors have shown that α-MSH, ACTH, and ORG2766, ([Met$(O_2)^4$,D-Lys^8,Phe^9]$ACTH_{4-9}$), have effects on nerve regeneration[113-116]. Specifically the $ACTH_{4-9}$ analogue ORG2766 is known to accelerate and enhance recovery of damaged peripheral nervous tissue[113]. Electrophysiological studies revealed that ORG2766 increased the speed of muscle contraction and decreased muscle fatigue during periods of prolonged stimulation[117]. The amino acid sequence of the $ACTH_{4-9}$ core contains the essential information for acceleration of functional recovery after nerve damage. ORG2766 did not compete with [Nle^4,D-Phe^7]α-MSH for binding in the CNS, implying the existence of further melanocortin receptors to mediate the effects of this peptide[118].

5) Effect on temperature control

α-MSH and $ACTH_{1-24}$ have been reported to lower core temperature of febrile rabbits when given peripherally or centrally[119]. Centrally administered α-MSH was 25000 times more potent than acetaminophen (paracetamol) in reducing fever in febrile rabbits[67]. α-MSH_{11-13} was reported to not only to reduce fever, but also possess anti-inflammatory actions. However, it was much less potent than the full-length peptide α-MSH[120]. The conformation of the C-terminal tripeptide of α-

MSH affects its activity. D-substitutions in position Ac-[D-Val^{13}]α-MSH$_{11-13}$ and Ac-[D-Lys^{11},D-Val^{13}]α-MSH$_{11-13}$ increase the antipyretic actions and anti-inflammatory, but Ac-[D-Pro^{12}]α-MSH$_{11-13}$ leads to loss of its anti-inflammatory effects[121]. Recently, a study demonstrated α-MSH was released in normal human subjects with high fever induced by endotoxin[122]. Endotoxins are components of the bacterial cell wall which reach the circulation during bacterial infection. The injection of endotoxin causes a number of physiological changes such as increasing fever.

6) Anti-inflammatory activity

α-MSH is a potent suppresser of inflammation, acting by down-regulating the activity or production of many cytokines. Administration α-MSH into mouse inhibited the peripheral inflammatory actions of the mediators, IL-1β , IL-6, and tumour necrosis factor (TNF)[123-125].

α-MSH inhibited the inflammatory mediator nitric oxide (NO) in cultured murine[126] and human macrophages[125] via inhibition of NO synthase II mRNA. MC1-R were found on cultured murine and human macrophages[126,127]. α-MSH is released by macrophages in response to cytokine treatment. α-MSH binds with MC1-R on macrophages, where it stimulates intracellular cAMP and inhibits NO production in monocytes[126,127]. α-MSH exerts its protective action against liver damage by binding MC1-R on mouse hepatic macrophages. MC1-R have been found in human neutrophils, and α-MSH has been reported to induce inhibition of neurophil migration to inflammatory sites[128]. Stimulation of Melanocortin receptor (MC receptors) inhibits cytokine and mRNA accumulation (including TNF, IL-8,

MCP-1) resulting in a reduction in the release of cytokines. This results in a decrease of neutrophil and macrophage host cell infiltration into the liver and inhibition of liver injury[129]. α-MSH and α-MSH_{11-13} are able to promote IL-10 production[130]. IL-10 inhibits the production of proinflammatory and immunomodulating cytokines, caused by binding of α-MSH with MC1-R to human monocytes. [Nle^4,D-Phe^7]α-MSH does not effectively block IL-1 action on thymocytes[123], suggesting that the immunomodulatory effects of α-MSH may not be mediated by the classic melanocyte α-MSH receptor (MC1-R).

7) Effect on cardiac contractability

In conscious rats, iv. administered $ACTH_{4-10}$ and γ_2-MSH induced a dose-dependent increase in blood pressure (BP), heart rate (HR) and pulse pressure (PP). α-MSH only caused an increase in HR, whereas the stable $ACTH_{4-9}$ analogue,ORG2766, was without effect on BP and HR[61].

1.5. Structure-Activity Relationships

The principal aim of this study is the design of hormone analogues with features of practical utility for medical application, such as increase potency, greater metabolic stability, increased selectivity, prolonged time course of action or ability to inhibit the natural hormone (antagonistic properties). Structure activity studies have proved to be very useful for the elucidation of the mechanism of hormone-receptor interactions. Since a peptide hormone usually has more than one

physiological role, the investigation within the peptide should precede any further study on the transmission of hormonal information from the peptide to the cell.

To date, most studies have been carried out with α-MSH and α-MSH analogues on the MC1-R, and were reviewed by Eberle up to 1988[39].

1.5.1. Core sequence: Glu-His-Phe-Arg-Trp

His-Phe-Arg-Trp (α-MSH_{6-9}) is found in all naturally occurring melanocortin peptides and because of its importance for biological activity, therefore, it is considered to be the core sequence of melanocortin peptides. In general, replacement of naturally occurring L-amino acids within this "core sequence" with D-amino acids led to a significant decrease in potency with the exception[131] of [D-Phe^7]α-MSH or [D-Phe^7,D-Trp^9]α-MSH, where the potency is increased by a factor of about 10. Recently, Ac-D-Phe-Arg-D-Trp-NH_2 has been reported as the most potent tripeptide in sustained melanotropic activity in frog skin[132]. Replacement of Arg^8 by D-Arg reduced the melanotropic potency[133]. Thus, both Phe^7 and Arg^8 are considered to be particularly sensitive to alterations and probably play a key role in eliciting the MSH response.

Glu^5 residue is the least important for melanotropic activity in the (5-9) core of α-MSH[39]. Replacement of Glu^5 with alanine did not significantly alter the activity of α-MSH at neither MC1-R nor MC3-R[134,135].

1.5.2. N-Terminal Ser-Tyr-Ser-Met:

Structure-activity studies show that receptors on different melanophores exhibit slightly different recognition patterns for linear α-MSH peptides, i.e. certain residues are more crucial for biological activity in some bioassays than in others. For example, the Ser-Tyr-Ser segment is important for stimulation of *Rana* MSH receptors but dispensable for eliciting the response in *Xenopus*[39].

Acetylation at the N-terminal protects the molecule from enzymatic degradation, and increased activity is due to the prolonged half life[136] .

Ser-Tyr-Ser is a hydrophilic part of the peptide. If these residues were substituted with lipophilic residues, a decrease in activity is seen[39]. Iodination at the [Tyr^2] will increase the lipophilicity, so the potency of [^{125}I-Tyr^2]α-MSH is slightly lower than that of α-MSH by about 2-fold[137]. H-Ser-Tyr-Ser-Met is inactive but on addition of H-Ser-Tyr-Ser-Met to α-MSH_{5-13} the potency increases 50-100 fold[44].

The Met^4 residue is important since oxidation of methionine to methionine sulphoxide led to a large decrease in potency (10-100 times) for the hormone. Substitution of Met^4 by the isosteric norleucine Nle^4 results in analogues that are resistant to oxidation and led to an increase in potency[23,138]. Replacement of Met with cysteine, which is structurally related, is possible, but replacement with alanine is not possible[134]. Some reports demonstrate that Ser-Tyr-Ser- is relatively unimportant to the overall biological activity and binding affinity of the native hormone[139,140], while α-MSH_{4-13} is as active as α-MSH.

1.5.3. C-Terminal Gly-Lys-Pro-Val

Gly^{10} has an important spacer function between the central and C-terminal hormonal active sites[141]. Alteration of Lys^{11} in the tripeptide α-MSH_{11-13} inactivates the fragment if the replacing residue is lipophilic or does not contain a basic side-chain. A change to [D-Pro^{12}] abolishes activity. Lys^{11} can be replaced by alanine without effect[134], attachment of a photoaffinity label in this position[135,162] has no effect as long as position 12 is not changed at the same time[134]. Modification at Val^{13} does not influence the potency of the peptides as long as the side-chain remains lipophilic[39].

Addition of Lys-Pro-Val-NH_2 to the C-terminus of the core heptapeptide resulted in a 1000 times increase in activity[39], suggesting that the tetramer 10-13, Gly-Lys-Pro-Val, is an important second hormonal 'active site' of α-MSH[39]. Some reports[139,140,142,143] found that addition of Lys^{11}, and Pro^{12} residues to cyclic melanotropin greatly enhanced biological activity. For example: [Cys^4,Cys^{10}]α-MSH exhibits potency >10000 α-MSH activity in the frog skin bioassay, while [Cys^4,Cys^{10}]α-MSH_{4-10} was found to be less active than α-MSH in frog skin assay, though [Cys^4,Cys^{10}]α-MSH_{4-13} was equipotent to [Cys^4, Cys^{10}]α-MSH[139]. This also demonstrated that N-terminal, Ser-Tyr-Ser-, is not important to whole sequence.

Incorporation of the Val^{13} residue results in no increase of potency. It is clear that the 4-12 sequence is the minimal sequence required for maximum potency[140,142].

1.5.4. [Nle^4,D-Phe^7] α-MSH

Melanotropic activities of synthetic stereoisomers of His-Phe-Arg-Trp-Gly showed that analogues replaced with D-Phe, D-Trp, or both, appear to have higher activity than the all-L-pentapeptides[131,133] in skin bioassays. [Nle^4,D-Phe^7]α-MSH is a "superpotent" agonist of α-MSH and is 26 times as potent as α-MSH in the mouse melanoma adenylate cyclase assay[23]. A single injection of [Nle^4,D-Phe^7]α-MSH into frogs caused near maximum darkening of the skins of the frogs for at least 6 weeks[26]. Injections of the natural hormone α-MSH or [Nle^4]α-MSH also caused darkening, but this effect lasted only a few days[26]. Catt *et al.*[144] have suggested that initial hormone interaction with receptor can effect a conformational change at the receptor resulting in tighter binding of the hormone to the receptor. [Nle^4,D-Phe^7]α-MSH may also induce tighter (apparently irreversible) binding to its receptor, leading to a sustained, biological activation of adenylate cyclase and cAMP production[26,145].

[D-Phe^7]α-MSH has been shown to protect the peptide from chemical degradation during *in vitro* and *in vivo* conditions. This would explain the higher prolongation as well as potency observed with [D-Phe^7]α-MSH analogues to the [L-Phe^7]α-MSH[26,138,146]. [Nle^4,D-Phe^7]α-MSH$_{4\text{-}11}$ has similar properties to [Nle^4,D-Phe^7]α-MSH in that it has high affinity, and in the tyrosinase assay the compound was 100-fold more active than α-MSH[147]. [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH is more stable to enzymatic degradation and is more resistant to oxidative conditions found during radioiodination. Therefore it is routinely used as a standard in biological assays and facilitates radioiodination for use in binding assays.

1.5.5. Cyclic Peptides

Since linear peptides can adopt a multitude of conformational states, restriction of conformational flexibility is a prerequisite for studying the "receptor-specific" conformation of the peptide. The only practical way to obtain semi-rigid analogues of peptides is by cyclization of suitable linear derivatives. It has been suggested that a β-turn or other peptide chain-reversal region within the central active site His-Phe-Arg-Trp of α-MSH might be functionally related to its biologically active conformation[39]. To evaluate the effect of covalently locking α-MSH into a reverse-turn conformation, Sawyer *et al.*[148] substituted cysteine residues for Met^4 and Gly^{10}, and oxidised the intermediate free disulfhydryl analogue to its intramolecularly disulfide-bridged derivative, the cyclic analogue Ac-[Cys^4,Cys^{10}]α-MSH. They reported that it had superpotent bioactivity in the frog skin bioassay but lacked significant prolonged activity[139,148]. Ac-[Cys^4,Cys^{10}]α-MSH has shown similar affinity as α-MSH in B16 murine melanoma cells expressing the MC1-R. Ac-[Cys^4,Cys^{10}]α-MSH had significantly higher affinity than its linear counterpart although the activities of the linear and cyclic analogues were similar[149].

Al-Obeidi *et al.*[150,151] designed cyclic lactam analogues of α-MSH resulting in superpotency and prolongation in lizard skin. The most active compounds were Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH_{4-10} and Ac-[Nle^4,Asp^5,D-Phe^7, Lys^{10}, Gly^{11}] α-MSH_{4-13}. Recently, Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 has been shown to have higher affinity and more prolonged activity compared to [Nle^4,D-Phe^7]α-MSH, indicating that the available conformations of the peptide can increase

receptor-ligand binding affinity and induce a receptor conformational change that effects the generation of cAMP by the same relative orders[152,153].

1.5.6. Antagonists

A specific, competitive and potent antagonist would be a useful guide to understanding interactions of α-MSH with its receptors, but how α-MSH specifically interacts with its receptor to trigger activation or which regions of the molecule should be modified to produce antagonist action is as yet unclear. The mechanism of α-MSH antagonist action can be studied by evaluating large numbers of structural variants of the molecule. Structure modification of α-MSH by substitution or deletion of certain amino acids has been used as an approach to identify α-MSH antagonists in various melanocyte bioassays. However, in previous studies, α-MSH antagonists have either not been highly potent or were agonists at high concentrations. Thus, much less has been achieved on the design of competitive antagonists of α-MSH.

Al-Obeidi *et al.* have synthesised a selective antagonist for the frog skin receptor, Ac-Nle-Asp-Trp-D-Phe-Nle-Trp-Lys-NH_2. Castrucci *et al.*[154] demonstrated that H-His-D-Arg-Ala-Trp-D-Phe-Lys-NH_2 was an antagonist *in vivo* in the lizard skin assay although in both cases the concentration of antagonist was very high in the 10^{-5} M range.

Recently, a series of potent antagonists of α-MSH has been published by Jayawickreme *et al.*[155]: Met^5-Pro^6-D-Phe^7-Arg^8-D-Trp^9-Phe^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH_2 (153N-6) and Ala^5-Leu^6-D-Phe^7-Arg^8-D-Trp^9-Phe^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH_2,

both inhibited the activity of α-MSH as measured in the c-AMP assay. Most antagonists contained the [D-Phe-Arg-D-Trp]α-MSH_{7-9}, and L-Phe (corresponding 10 position of α-MSH) may be important for antagonist activity. The peptide, 153N-6, could competitively inhibit the binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH binding at every melanocortin receptor albeit with different potency[156]. The shorter tripeptide, D-Trp-Arg-Leu-NH_2, was reported[157] to antagonise the human melanoma MC1-R with an IC_{50} of 0.6μM. Some antagonists were found by Adan *et al.*[158] and tested on rat MC3, human MC4 and ovine MC5 receptors with alteration in the $ACTH_{4-10}$ positions. The structures used were [Phe-I^7]-$ACTH_{4-10}$, which antagonised all three receptors although activity was very low *in vitro*; and [D-Arg^8]-$ACTH_{4-10}$, [$Pro^{8,10}$,Gly^9]-$ACTH_{4-10}$ antagonised the MC4-R and MC5-R. Hruby *et al.*[159] suggested that modifications of the phenyl ring of the D-Phe^7 residue of a cyclic lactam derivative, while retaining the aromatic character, can result in melanocortin receptor antagonists with high potency and specificity and the activity was found to be very low *in vitro*. In this study, naphthylalanine was introduced in position 7 to give the cyclic lactam peptide Ac-Nle^4-[Asp^5,(D-Nal(2)^7,Lys^{10}]α-MSH_{4-10}-NH_2(SHU9119). This was a potent antagonist of MC4-R and less potent antagonist of MC3-R but a full agonist of the MC1-R and MC5-R. Likewise, substitution with [D-p-iodophenylalanine^7] to Ac-Nle^4-[Asp^5,D-Phe (pI)^7, Lys^{10}]α-MSH_{4-10} was found to be a potent agonist at the cloned human MC1-R and mouse MC1-R but had potent antagonist activities at the human MC4-R and human MC3-R.

1.6. MSH Receptors

There are numerous targets for drugs, the most common being enzymes, receptors, ion channels, and active transport complexes. Melanocortins exert their effects by binding to G-protein-coupled cell surface receptors and couple with the adenylate cyclase second messenger, cAMP (see section 1.3.1.). Since 1992, molecular cloning identified a family of G-protein coupled receptors that bind and are stimulated by melanocortic peptides. Up until now, five melanocortin receptors have been identified; the melanocyte MSH receptor MC1, and the adrenal ACTH receptor MC2 are located in the periphery, while MC3, MC4 and MC5 are localised mainly in the brain. MC3 is situated in the hypothalamus. MC4 is widespread in the brain and MC5 has not yet been mapped sufficiently.

1.6.1. MC1 Receptor

The first melanocortin receptors cloned were the mouse and human melanoma MSH receptors[15,16]. The mouse receptor is 315 amino acids long and human receptor is 317 amino acids. The Human MC1-R is 76% homologous with the mouse MC1-R. Transfection of human embryo kidney 293 cells with mouse MC1 cDNA results in the expression of a functional MC1-R. The measurement of cAMP production following stimulation with MSH peptides revealed the activity profile: [Nle^4,D-Phe^7]α-MSH> α-MSH= β-MSH>ACTH. Moutjoy *et al* showed that γ-MSH was not able to cause cAMP production[15,53]. L cells, which are derived from murine fibroblasts, transfected with human MC1-R gave a slightly different result: [Nle^4,D-Phe^7]α-MSH> α-MSH = ACTH> β-MSH>> γ-MSH[17]. Similar

results apply to human melanocytes naturally expressing MC1-R as shown by c-AMP assays and competitive binding[160]. In human melanocytes, γ-MSH increased cAMP production, although it was not a full agonist[17]. Competitive binding (displacement of [^{125}I]NLDP-MSH by other melanocortins) in COS-7 cells transfected with the human MC1-R cDNA showed that [Nle^4,D-Phe^7]α-MSH> α-MSH> ACTH> β-MSH>γ-MSH>> $ACTH_{4-10}$[16] and human MC1-R expressed in COS cell display [Nle^4,D-Phe^7]α-MSH> α-MSH> desacetyl-αMSH> $ACTH_{1-10}$> β-MSH> γ_3-MSH>ACTH > γ_1-MSH> γ_2-MSH> $ACTH_{4-10}$[18].

Northern blot analysis showed that the MC1-R is naturally expressed in most melanoma cell lines[15,16] and in cultured murine and human macrophages[126,127]. Moreover, an MC1-type receptor isolated from primary rat Schwann cells has been reported[161] detected by photoaffinity labelling with [^{125}I-Tyr^2,Nle^4,D-Phe^7, ATB-Lys^{11}]α-MSH. In this study it was shown that the molecular weight of the Schwann cell receptor was 42-45 kDa, similar to that found in the B16 melanoma cell line, 43-46 kDa, and was thought to be an MC1-R[162]. The functions of the MC1-R are considered to be pigment dispersion in melanocytes and modulation of anti-inflammatory activity in monocytes[126,127].

1.6.2. MC2-Receptor

The MC2-R, specific for ACTH, is expressed only in the adrenal gland[15]. The gene encoding the mouse MC2-R was recently cloned and shown to possess 89% amino acid sequence homology with the human MC2-R, the mouse ACTH receptor is 296 amino acids long[163], and shows, 38,42,46,44% amino acid homology with the

MC1-R, MC3-R, MC4-R, and MC5-R, respectively[164]. ACTH was reported to induce up-regulation of both ACTH receptor transcripts and ACTH receptor numbers in cultured human adrenocortical cells[165]. Some studies[164,166] demonstrated that ACTH receptor specifically binds ACTH but not other melanocortin peptides, explaining why the MSH peptides are not known to induce steroidogenesis.

1.6.3. MC3-Receptor

The MC3-R is found in brain and placenta but not in melanoma cells or adrenal tissue[17]. The cloned human receptor is 361 amino acids long[17], and the rat and mouse receptor 323 amino acids[62,63]. It has been confirmed by Desarnaud *et al* the difference between human and mouse receptor is 37 amino acids[63]. Despite the 37 amino acid difference, mouse and human receptors are 88% homologous. The amino acid sequence of MC3-R is 43-46% homologous with that of MC1-R or MC2-R. The activity profile of melanocortin peptides at the MC3-R on cAMP accumulation is as following: α-MSH= β-MSH= γ- MSH= ACTH[17], [Nle^4,D-Phe^7]α-MSH> γ_1-MSH= γ_2-MSH= α-MSH= $ACTH_{(1-39)}$> γ_3-MSH> desacetyl α-MSH>> $ACTH_{4-10}$[62], and [Nle^4,D-Phe^7]α-MSH= γ_2-MSH> β-MSH= αMSH>$ACTH_{1-39}$ >> $ACTH_{4-10}$[63]. The competitive binding assay shows that [Nle^4,D-PHe^7]α-MSH> desacetyl-αMSH> γ_1-MSH> γ_3-MSH> β-MSH> γ_2-MSH> α-MSH> ACTH> $ACTH_{1-10}$> $ACTH_{4-10}$[18]. The order of activity differs for α-MSH but all authors agree that the MC3-R responds relatively strongly to γ-MSH, and all reports show that certain brain areas express this receptor[17,62,63]. The MC3-R

does not bind ORG2766. The inactivity of ORG2766 means that the MC3-R is not likely to be involved in neural regeneration[62]. It has been suggested that MC3-R might be specific for γ-MSH[62] and may be involved in cardiovascular function. However, several reports have shown that neither MC3-R nor MC4-R is involved in the centrally mediated presser activity of γ-MSH[57,167,168] so the main function of the MC3 receptor is still unknown.

1.6.4. MC4-Receptor

Human MC4-R are located mainly in the brain[169,170]. The human MC4-R is 333 amino acids long[170] and the rat MC4-R is 332 amino acids long[169]. They have approximately 95% homology. The MC4-R is most closely related to the other two newly described melanocortin receptors, MC3-R[17,62] and MC5-R[171-174], with 55-61% homology, demonstrates slightly less homology (46-47%) with MC1-R and MC2-R. The activity established by cAMP accumulation caused by different peptides at MC4-R are the following: [Nle^4,D-Phe^7]α-MSH> desacetyl-α-MSH>/ACTH>/α-MSH>> γ_2-MSH> $ACTH_{4-10}$[169]. α-MSH= ACTH= β-MSH>> γ-MSH >$ACTH_{1-10}$>$ACTH_{4-10}$[170]. The potency in the binding assay shows the following profile: [Nle^4,D-Phe^7]α-MSH> [Nle^4]α-MSH > β-MSH> desacetyl-α-MSH>α-.MSH> ACTH> $ACTH_{4-10}$>γ_1-MSH> γ_2-MSH[175]. Among the endogenous MSH peptides, β-MSH shows the highest affinity for MC4-R[175]. It has therefore been suggested that β-MSH might be the natural ligand for this receptor[175].

Ligands binding to MC4-R induce excessive grooming behaviour in rats, and the potency for excessive grooming behaviour via MC4-R is: [Nle^4,D-Phe^7]α-MSH>

α-MSH> $ACTH_{(4-13)}$[158,167]. The MC4-R is more widely distributed in the central nervous system than the MC3-R and is represented at multiple sites in almost every brain region. Like the MC3-R, MC4-R does not respond well to $ACTH_{4-10}$ and does not respond at all to ORG2766. These compounds are more active than native melanocortins in behavioural assays involving the retention of acquired behaviour[113] and they enhance nerve regeneration[114]. Thus, neither MC3-R nor MC4-R appear to be involved in nerve regeneration and acquired behaviour. Recently, some reports have shown that mouse agouti peptide displayed antagonist activity at MC4-R[86,89]. Intracerebroventricular administration of agouti-mimetic peptide, Ac-Nle^4-c[Asp^5,(D-Nal(2)^7,Lys^{10}]α-MSH_{4-10}-NH_2 (SHU9119), was shown to be a potent antagonist of MC4-R[159], and ,like the agouti peptides, was able to enhance food intake significantly. This indicates that the MC4-R might be involved in the control of feeding behaviour.

1.6.5. MC5-Receptor

The MC5-R has been cloned from genomic libraries of different species, human[174,176], ovine[177], mouse[171,172], rat[173]. In all species the predicted receptor is 325 amino acids in length and the amino acid sequence shows 42%-50% homology with MC1-R, 45% with MC2-R, 53-69% with MC3-R, and 62% with MC4-R. MC5-R has been detected in skeletal muscle, adrenal gland, brain, and wide variety of peripheral tissues[172-174,177]. The potency with which the MC5-R responds to melanocortin peptides with c-AMP accumulation is the following: [Nle^4, D-Phe^7]α-MSH> α-MSH> $ACTH_{(1-39)}$ = β-MSH= γ_1-MSH > γ_2-MSH= γ_3-

MSH[171], [Nle^4,D-Phe^7]α-MSH= α-MSH= $ACTH_{1-24}$> ACTH= β-MSH> γ-MSH>> $ACTH_{4-10}$[173], [Nle^4,D-Phe^7] α-MSH> $ACTH_{1-24}$> α-MSH> β-MSH> γ-MSH[174], α-MSH> β-MSH> ACTH> γ-MSH[172], [Nle^4,D-Phe^7] α-MSH= α-MSH= β-MSH[177]. Competitive binding assays show the following activities: [Nle^4,D-Phe^7]α-MSH> α-MSH= ACTH> β-MSH> γ-MSH[176], [Nle^4,D-Phe^7] α-MSH> α-MSH> β-MSH> ACTH> γ_3-MSH> γ_1-MSH> γ_2-MSH[171], [Nle^4,D-Phe^7] α-MSH> α-MSH= ACTH> β-MSH[177]. [Nle^4,D-Phe^7]α-MSH > desacetyl-α-MSH> α-MSH>β-MSH> ACTH>γ_1-MSH[18]. MC5-R differs from MC1-R, MC2-R, MC3-R , and MC4-R by its presence in skeletal muscle, in addition to brain and adrenal gland.

1.6.6. Differences Between MC1 and MC3 Receptor

MC1 receptors appear to occur primarily in peripheral cells. For example, MC1-R were found in mouse and human melanocytes and melanoma cells, and in murine and human macrophages[126,127]. MC1-R shows high and selective affinity and activity for α-MSH, but γ-MSH peptides, which are predominant in human pituitary[55], have very low affinity and activity at MC1-R[15,17]. This indicates that γ-MSH has no function in pigmentation. Ling *et al.*[55] demonstrated that γ_1-MSH, γ_2-MSH, or γ_3-MSH were unable to stimulate the melanocytes in the *Rana Pipiens* melanophore assay except at very high concentrations compared to α-MSH. Due to the localisation of MC1-R, this receptor may mediate skin pigmentation effects and anti-inflammatory activities of α-MSH.

MC3-R are found in brain and placenta but not in melanoma cells or adrenal tissue[17]. The distribution of γ-MSH immunoreactivity corresponds well with that of MC3-R mRNA expression in brain, and γ-MSH possess a higher affinity and activity for MC3-R than for the MC1-R, MC3-R has thus been implicated in the control of cardiovascular function[17,62]. The pharmacological profile of the MC3-R *in vitro* is not consistent with the *in vivo* cardiovascular actions of γ_2-MSH[57,61,167,168]. $ACTH_{4-10}$ showed no affinity or activity at MC3-R, suggesting that MC3-R might not have an effect on nerve regeneration in the peripheral nervous system or induction excessive grooming behaviour[167].

1.6.7. Summary

The main characteristics of the five MC receptors are: MC1-R is a specific α-MSH receptor expressed only in melanoma cells and melanocytes and macrophage, while MC2-R is an ACTH receptor expressed in the adrenal gland. MC3-R, MC4-R, and MC5-R are detected in brain tissue. MC3-R differs from MC4-R and MC5-R as it specifically recognises peptides with the $ACTH_{4-10}$ core sequence. MC5-R differs from MC3-R and MC4-R in its pattern of expression. Besides the brain, MC5-R is also found in the adrenal gland and skeletal muscle. Which MC receptor is involved in the neurotrophic and neuroprotective actions of melanocortins is not yet clear. The most promising candidates are the MC3-R, MC4-R, and MC5-R, as they are expressed in brain and peripheral tissues. The MC5-R may be responsible for the effects of melanocortins on regeneration and development in muscle, as this is the only member of the MC receptor family present in skeletal muscle[171,172,174].

A problem is that $ACTH_{4-10}$, which is fully active in peripheral nerve regeneration, is much less potent than α-MSH or has no effect on the cAMP production in cells transfected with the MC3-R[62], MC4-R[168], and MC5-R[171]. Thus, it is possible that fewer melanocortin receptors exist. An overview of the pharmacological data published for melanocortin receptors is given in Table.1.1.

R	species	Cell	Efficacy Order of Melanocortin	No Effect	Ref
1	mouse	human 293	A:NLDP>α-MSH=β-MSH=ACTH	γ-MSH	15
1	human	COS7	B:NLDP>α-MSH>ACTH>β-MSH>γ-MSH>$ACTH_{4-10}$	β-endorphin	16
1	human	murine L	A:NLDP>α-MSH=ACTH>β-MSH>>γ-MSH		17
1	human	COS	B:NLDP>α-MSH>desacetyl α-MSH>$ACTH_{1-10}$ >β-MSH >γ_3-MSH >ACTH>γ_1-MSH>γ_2-MSH>$ACTH_{4-10}$		18
3	human	murine L	A:NLDP=α-MS=β-MSH=γ-MSH=ACTH>$ACTH_{1-10}$ > $ACTH_{4-10}$		17
3	rat	human 293	A:NLDP>γ_2-MSH=γ_1-MSH=α-MSH=ACTH>γ-MSH > desacetyl α-MSH>>$ACTH_{4-10}$ B:NLDP>γ_2-MSH=α-MSH>>$ACTH_{4-10}$	ORG2766 ORG2766	62
3	mouse	CHOK	A:NLDP=γ_2-MSH>β-MSH=α-MSH>ACTH>>$ACTH_{4-10}$	β-Endoephin	63
3	human	COS	B:NLDP>desacetyl α-MSH>γ_1-MSH>r_3-MSH>β-MSH >γ_2-MSH>α-MSH>ACTH>$ACTH_{1-10}$>$ACTH_{4-10}$		18
4	rat	human 293	A:NLDP>desacetyl α-MSH>/ACTH>/α-MSH>>γ_2-MSH =$ACTH_{4-10}$	ORG2766	169
4	human	murine L	A:α-MSH=β-MSH=ACTH>>γ-MSH=$ACTH_{1-10}$ =$ACTH_{4-10}$		170
4	human	COS	B:NLDP>[Nle^4] α-MSH>β-MSH>desacetyl α-MSH>α-MSH>ACTH>$ACTH_{4-10}$ >γ_1-MSH>γ_2-MSH		175
5	mouse	CHO	A:NLDP>α-MSH>ACTH=β-MSH=γ_1-MSH>γ_2-MSH=γ_3-MSH B:NLDP>α-MSH>β-MSH>ACTH>γ_3-MSH>γ_1-MSH>γ_2-MSH	β-Endorphin β-Endorphin	171
5	rat	CHO	A:NLDP=α-MSH=$ACTH_{1-24}$>ACTH=β-MSH>γ MSH>>$ACTH_{4-10}$		173
5	human	COS1	A:NLDP>$ACTH_{1-24}$>α-MSH>β-MSH>γ-MSH		174
5	mouse	mouse L	A: α-MSH>β-MSH>ACTH>γ-MSH A: α-MSH>$ACTH_{1-10}$>$ACTH_{4-13}$		172
5	ovine	COS7	B: NLDP>α-MSH=ACTH>β-MSH A:NLDP=α-MSH=β-MSH	γ-MSH β-Endorphin	177
5	human	COS7	B:NLDP>α-MSH=ACTH>β-MSH>γ-MSH		176
5	human	COS	B:NLDP>desacetyl α-MSH>α-MSH>β-MSH>ACTH>γ_1-MSH		18

Table 1.1.: Functional Coupling of Melanocortin Rs to Adenylate Cyclic AMP and Binding. A: c-AMP assay, B: competitive binding assay, NLDP: [Nle^4,D Phe^7] α-MSH. The activities and affinities observed were measured after transfecting cells with the receptors.

1.7. Aims and Objectives

The long-term aim of this study is to characterise promising approach to the selective drug treatment for malignant melanoma. Melanoma is notorious for its difficulty of detection and its tendency to metastasise at an early stage. So far, the treatment for melanoma is still limited to surgery, which is only possible at early stage. In this study, we focus on the study of α-MSH and related peptides as putative candidates for a more selective treatment of malignant melanoma.

α-MSH and its analogues are found in the brain as well as in peripheral tissues. Therefore, the functions of α-MSH not only have great impact in pigmentation but the peptides possess many CNS effects such as influence on learning, attention, and memory, anti-inflammatory, cardioacceleration, nerve regeneration, and antipyretic. Since so many physiological actions are displayed by α-MSH and its analogues, there must be multiple and specific receptors to mediate these various effects. The existence of several different melanocortin receptors could be exploited when trying to target specific type receptors at present only in the cell type of interest.

Up to date, five melanocortin receptors have been cloned. The specific functions of MC1-R and MC2-R are known to be pigmentation and anti-inflammatory activity, and steroidogenesis respectively. The functions of the other receptors are still uncertain.

In this study, I focus on the relation of the α-MSH and its analogues with different receptors, in particular the MC1-R from B16 murine melanoma cells and the MC3-R from rat hypothalamus as tested by binding assay and cAMP accumulation.

Several approaches have been attempted to achieve the discovery of selective ligands for melanocortin receptor subtypes:

Position 12 of α-MSH (Proline) has been suggested to be an important position for its activity. We have attempted to modify this position to test how it affects the activity at MC1-R and MC3-R as well as corresponding modifying position in γ_1-MSH peptides.

[D-Phe^7]α-MSH is very important to enhance its prolongation and potency. A recent paper has suggested that substitute [D-Phe^7]α-MSH residues with bulky aromatic amino acids may act as potent and receptor selective antagonist. Thus, alterations of [D/L-Tyr^7]α-MSH and [D/L-Trp^7]α-MSH have been made to investigate antagonist activity at MC1-R, MC3-R and MC4-R.

Cyclic peptides have been proposed to be more stable and have higher activity than linear ones. Several Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$ analogues have been synthesised and tested their affinity and activity at MC1-R.

Chapter 2: Materials and Methods

2.1. Peptide Synthesis

2.1.1. Materials for Peptides Synthesis

α-MSH and γ_1-MSH were obtained from Bachem, Switzerland. Alanine-substituted peptides and analogues modified in position 7 were synthesised by Dr. U.G.Sahm in our laboratory and cyclic peptides by Dr. S.Bansal at Pharmacy, King's college, London. All amino acids, Polydimethylacrylamide-Kieselguhr resin (Pepsyn K) and p-(R,S-L-1(^{9}H-fluoren-9-yl)methoxy-formamide-2,4-dimethoxybenzyl) phenoxyacetic acid (AM-linker) were obtained from MilliGen, Watford.
Hydroxybenzotriazole (HOBT), trifluoroacetic acid (TFA), diisopropylcarbodiimide (DIC), 1,2-ethanedithiol (EDT), anisole and thioanisole were from Aldrich, Gillingham, Dorset. Activated esters used were pentafluorophenyl esters (PFP), except for serine which was used as 3,4-dihydro-4-oxyobenzotriazin-3-ylester (ODhbt), all amino-protecting group were Fluorenylmethoxycarbonyl (Fmoc). Purified peptides were stored at -20^{0}C, and peptides on resin were kept at 4^{0}C.
All peptides were synthesised using a MilliGen 9050 pepsynthesizer. The column for analytical HPLC was 250mm X 4.9mm, and for preparative HPLC 250mm X 25mm; they were obtained from LKB and Waters, respectively. The Packing material for both HPLC was Vydac 5 μ C18 300A pore size.

2.1.2. Methods

2.1.2.1. Peptide Synthesis:

After peptide synthesis using the automatic synthesizer, resin was washed with dichloromethane to remove impurities and dimethylformamide (DMF), followed by methanol and diethylether washes. The washed resin was under dried N_2.

2.1.2.2. Deprotection and Cleavage from Resin:

The peptide was deprotected and cleaved from the resin using 90% TFA, 5% ethanedithiol, 2% anisole and 3% thioanisole, over a period of 8 hours as determined by preliminary experiments. The peptide was then washed with petroleum ether to remove TFA and scavengers, and precipitated with diethyl ether. The crude peptide was redissolved in distilled water and freeze dried.

The crude peptide was analysed by analytical grade HPLC using a gradient elution with 0.1% TFA in water/ 90% acetylnitro (CAN), 0.1% TFA in water at 1% per min. The eluent was monitored at 217nm.

2.1.2.3. Purification by Preparative HPLC

The crude peptide was redissolved in 3.5% ACN, 0.1% TFA and purified by preparative HPLC using the above gradient. Fractions were collected at appropriate elution times. Fractions were analysed by analytical HPLC; fractions containing the target peptide were pooled and freeze dried. Peptides were identified by mass spectrometry and their purity confirmed using capillary electrophoresis which is from BioRad.

2.1.3. Results for Peptide Synthesis:

All the peptides were tested by the mass spectrometry. FAB-MS was carried out at Swansea University SERC, and electrospray mass spectrometry were provided at University of Bath. Results are shown the Table.2.1.

The capillary electrophoresis showed only a single peak for each peptides.

Peptides	Molecular Weight Calculated	Found
[Nle^4,D-Phe^7]α-MSH	1646.8	1647.0
[Nle4,D-Phe^7,Phe^{12}]α-MSH	1696.9	1695.3
[Nle^4,D-Phe^7,Ser^{12}]α-MSH	1636.7	1635.4
[Nle^4,D-Phe^7,Leu^{12}]α-MSH	1663.9	1663.2
[Asp^{10}]α-MSH	1722.8	1721.1
[Phe^{12}]α-MSH	1715.9	1715.4
[Asp^{10},Phe^{12}]α-MSH	1772.9	1772.2
[Nle^3]γ$_1$-MSH	1494.0	1495.0
N$^{\alpha}$-Lys-[Nle^3]γ$_1$-MSH	1622.0	1623.0
[Nle^3,Pro^{11}]γ$_1$-MSH	1444.0	1444.0
[Nle^3,Ser^{11}]γ$_1$-MSH	1434.0	1434.0
[Nle^3,Leu^{11}]γ$_1$-MSH	1460.0	1461.0
[Ala^{11},Ala^{12}]α-MSH	1582.7	1580.7
[Ala^{11},Ala^{13}]α-MSH	1580.7	1579
[Ala^{12},Ala^{13}]α-MSH	1611.7	1610.1
[Ala^{11},Ala^{12},Ala^{13}]α-MSH	1554.6	1552.6
[L-Tyr^7]α-MSH	1681.8	1680.2
[L-Trp^7]α-MSH	1704.8	1703.2
[D-Tyr^7]α-MSH	1681.8	1680.2
[D-Trp^7]α-MSH	1704.8	1703.2
[Nle^4,cyc-Asp^5,D-Phe^7,cyc-Lys^{10}]α-MSH$_{(4-13)}$	1348.8	nd
[cyc-Asp^5,D-Phe^7,cyc-Lys^{10}]α-MSH$_{(5-13)}$	1235.7	1234.8
[Nle^4,cyc-Asp^5,D-Phe^7,cyc-Lys^{10}]α-MSH$_{(1-13)}$	1685.8	1685.0

Table.2.1: Mass Spectroscopy Data of the Peptides Synthesised
nd= not determined

2.2. Cell Culture

2.2.1. Materials

2.2.1.1. Water

Water for the preparation of all cell culture media and solutions was freshly double glass distilled using a bi-distillation Fistreem still fitted with a Fistreem predeioniser. (Fisons Ltd.)

2.2.1.2. Balanced Salt Solutions

Phosphate buffered saline (PBS) without divalent cations was obtained from Oxoid Ltd in tablet form. One tablet was dissolved in 100 ml of freshly double distilled water and autoclaved. Solutions were stored at 4 °C for up to four weeks.

2.2.1.3. Base and Acid Solutions

Solutions of 7.5% w/v sodium bicarbonate and 1 M sodium hydroxide were prepared using double distilled water and were steam sterilised. 0.1 mM HCl was prepared by diluting 1M HCl (BDH Laboratory Reagent Ltd.) in double distilled water followed by filter sterilisation. Solutions were kept at room temperature.

2.2.1.4. Ethylenediaminetetraacetic Acid (EDTA)

A 0.02% w/v solution of the disodium salt (BDH Laboratory Reagents Ltd.) was prepared in PBS, sterilised and stored at 4^0C , and made up monthly.

2.2.1.5. Trypan Blue

The stain was obtained from BDH Laboratory Reagents Ltd. and dissolved at 0.1 % w/v in PBS and filter-sterilised.

2.2.1.6. Growth Medium and Additives

RPMI 1640 (without L-glutamine and sodium bicarbonate) 10X, MEM non-essential amino acids 100X, L-glutamine 200mM, Penicillin (10,000 IU/ml) and streptomycin (10,000 mg/ml) were obtained from Gibco, Paisley, UK.

Batches of foetal calf serum were tested prior to routine use to ensure support of cell growth. Batches used were obtained from ICN and Gibco. All cells lines were cultured in RPMI 1640 medium supplemented with 10 % FBS, 200mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin, MEM non-essential amino acids and 7.5% w/v sodium bicarbonate. The culture medium was made up using the following formula:

Reagent	Volume (ml)
Double distilled water	450
RPMI 1640	50
Foetal Calf Serum	55
7.5%$NaHCO_3$	13.5
MEM Nonessential amino acids	5
Penicillin + Streptomycin	5
L-Glutamine	5
1 N NaOH	adjust to pH: 7.2-7.4

The pH was adjusted to 7.2-7.4 by the addition of an appropriate volume of 1N sodium hydroxide or 1M HCl. The medium was stored at 4^0C and used within two weeks. Culture of HEK cells transfected with the MC3 and MC4 receptors required addition of 0.5 mg/ml amphotericin B.

2.2.2. Laboratory Apparatus

All aseptic techniques were performed in a laminar flow cabinet (MDH Ltd.) with vertical recirculation. Cells were maintained at 37^0C in a LEEC PF2 anhydric incubator (Laboratory and Engineering Company). An inverted microscope, WILD M40 (Wild Heerbrugg Ltd.) was used for monitoring cell growth and determining cell numbers together with a standard double grid haemocytometer (Fisons Ltd.).

2.2.2.1. Disposable Items

Sterile tissue culture flasks were obtained from Falcon (Fahrenheit Labs, Bristol) and polypropylene ampoules for cell freezing from Corning, (Fahrenheit Labs.) 96- and 24- well culture plates were supplied by Nunc. 96-well filtration plates and filters were from Millipore.

2.2.2.2. Glassware

Used glassware was soaked in a 2 % v/v solution of RPS (Fisons) for 30 minutes with rinsing in three changes of single distilled water. Items were subsequently soaked in double distilled water for about 2 hours, dried in a hot air oven (Gallenkamp) capped with aluminium foil and oven sterilised (Gallenkamp) by dry heat at 160 °C for 1 hour.

2.2.3. Cell Culture

2.2.3.1. Cell Lines

A B16 murine melanoma cell line was donated by L.R.Kelland, Institute of Cancer Research, Sutton. Human 293 cells transfected with the genes for the expression of the MC3 and MC4 receptors, respectively were obtained from R.D.Cone, Vollum Institute, Portland, Oregon, USA.

2.2.3.2. Cell Storage and Recovery

Cells were stored in 2 ml ampoules in the vapour phase of a Union Carbide LR-40 liquid nitrogen refrigerator at approximately -148°C. Cells were prepared for storage by detaching confluent cells from the flasks using the method described for cell subculture. Cells were resuspended in culture medium, centrifuged for 10 minutes at 1000 rpm and resuspended in culture medium containing 10% dimethyl sulphoxide (Aldrich, spectrophotometric grade) as a cryprotectant. Ampoules were then placed in a Union Carbide BF6 biological freezer unit plug and put in a Union Carbide LR-33 liquid nitrogen refrigerator to allow them to cool below -70 °C at a rate of about 1 °C/ min^{-1}. They were then transferred to the liquid nitrogen refrigerator for long term storage.

To recover cells from storage, the cryotubes were placed in a 37 °C water bath and defrosted. Cells were suspended in 10ml of culture medium, centrifuged for 10 min at 1000 rpm, the cell pellet was resuspended and transferred to a 175 cm^2 tissue culture flask containing prewarmed medium.

2.2.3.3. Cell line Maintenance and Subculture

Cells were grown as monolayers in 175 cm^2 tissue culture flasks and examined daily for possible microbial contamination. The time to reach monolayer coverage varied between cell lines. In order to maintain a pH 7.2-7.4, the medium was routinely changed the day before the cells reached confluence. When cells reached confluence

(roughly $2x10^7$ cells/175cm^2), they were subculture by the monolayer twice with 5 ml PBS to remove traces of serum which would inhibit the action of EDTA. This was followed by a 10 minute incubation at 37^{0}C with 2 ml 0.02% w/v EDTA/PBS. Detached cells were diluted with culture medium to 10 ml, a sample was taken and counted and flask were inoculated with 2 x 10^6 cells per 175 cm^2 in new flask containing 50-75 ml of fresh medium. All cell lines were maintained in a LEEC anhydric incubator at 37^{0}C under standard conditions of 95 % air 5% CO_2 in a humidified atmosphere.

2.2.3.4. Determination of Cell Density

After detached the cells from the flasks, a 0.4 ml sample of the cell suspension was mixed with 0.1ml trypan blue solution. Viable cells exclude the dye, and nonviable cells are stained dark blue. A sample of the stained suspension was loaded into a grid haemocytometer under the coverslip. A count of the four corners and the central square was viewed with an inverted microscope, then the number of cells was calculated using the following equation.:

cells/ml = (Total cells in 5 chambers x 10^4)/4

2.3. Radioiodination at [Nle^4,D-Phe^7]α-MSH

Iodination was carried out at the [Tyr^2] position of [Nle^4,D-Phe^7]α-MSH by the oxidative chloramine-T method described by Eberle[39].

2.3.1. Solutions

- 1% Polypep: 0.05 g of polypep (Sigma) was dissolved in 1ml phosphate buffer, PH 7.4 and 4ml of Double distilled water (DDH) directly before use.
- 0.25%BSA: 0.0025g BSA (Sigma) was weighted and dissolved into 1ml phosphate buffer, PH 7.4 and 4ml of DDH directly before use.
- 0.1% Chloramine T: 0.01g of chloramine T (Fisons) was weighted and dissolved in 10ml of DDH directly before use.
- 0.25M Phosphate Buffer, PH 7.4: mixture of 0.25M Na_2HPO_4 and 0.25M NaH_2PO_4

  Na_2HPO_4 (BDH): 4.45g dissolved in 100ml DDH.

  NaH_2PO_4 (BDH): 3.90g dissolved in 100ml of DDH.
- The pH 7.4 of Na_2HPO_4 is adjusted by NaH_2PO_4. The stock solutions are stored at 4^0C and prepared monthly, and the phosphate buffer is prepared on the day of the iodination.
- 1%TFA: 1ml of pure TFA is diluted to 100ml DDH and stored at 4^0C.
- 80% MeOH + 1% TFA: 80ml methanol and 1ml TFA are diluted to 100ml DDH and placed at 4^0C. TFA was obtained from Alsrich.
- 60% MeOH + 1 % TFA: 60ml methanol and 1ml of TFA are diluted to 100ml DDH and stored at 4^0C.
- 50% MeOH + 1 % TFA: 50ml methanol and 1ml of TFA are diluted to 100ml DDH and placed at 4^0C. The MeOH is from Fisons.
-

2.3.2. Preconditioning of Purification Columns

A C18 reverse phase bond-elute column packed with Spherisorb ODS (Anachem) was preconditioned by washing according to the following protocol:

Step 1: 3 X 1ml 1% TFA

Step 2: 3 X 1ml 80% MeOH / 1% TFA

Step 3: 1 X 1 % polypep

Step 4: 3 X 1 ml 80% MeOH/1% TFA

Step 5: 3 X 1 ml 1% TFA

The last wash of 1% TFA on the top of the column to avoid "dry out"

2.3.3. Iodination

1.5 µl of 1mg/ml solution of [Nle^4, D-Phe^7]-αMSH is added to 20µl of 0.25M phosphate buffer, PH 7.4. Then,10µl of Na ^{125}I and 10µl of chloramine-T are added and allowed to react for 30 seconds before addition of 0.6ml BSA solution. The reaction mixture is transferred to the Bond Elut column to separate free iodine from mono-iodinated and di-iodinated [Nle^4, D-Phe^7]-α-MSH. The column is washed as following:

Step 1: 2 X 1ml 0.25 M Phosphate buffer

Step 2: 4 X 1ml 50%MeOH/1%TFA

Step 3: 2 x 1ml 60% MeOH/1%TFA.

The first 2ml of wash, are discarded. The remainder is purified by HPLC.

2.3.4. Purification of [Tyr^2,Nle^4,D-Phe^7]α-MSH

The methanol wash is purified by HPLC with an exponential gradient of 0.1 %TFA in water and 0.1%TFA in 70% Acetonitrile/ 30% water. 1ml/min fractions are collected

between 25 and 45 minutes after injection. The monoiodinated peptide elutes before the diiodinated derivative. The ^{125}I activity of each 1ml fraction is determined by transferring tubes to a LKB Wallac 1277 Gammamaster automatic gamma-counter and those with radioactivity associated with the peak of monoiodinated peptide are pooled and their is activity counted again. The radiolabelled peptide could be stored at -20^0C for up to 20 days.

2.3.5. Calculation of Radiotracer Concentration

According to the definition, 1 atom of ^{125}I associates with 1 mole [Nle^4,D-Phe^7]α-MSH to give 1 mole [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH.

Carrier-free Na^{125}I has a specific activity of 80.5×10^{15} Bq/atom (Amersham), so the activity of 1 mole [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH.= 80.5×10^{15} Bq.

1Bq= 1 decay per second, or 60 decays per minute and the efficiency of the gamma counter =70%

1 mole [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH would register $80.5 \times 10^{15} \times 60 \times 70\% = 3.38 \times 10^{18}$ cpm on the LKB 1277 Gammamaster.

2.4. Binding Assays

Binding assays were carried out following a method adapted from Siegrist et al.[179] and as described by Erskine-Grout[180].

2.4.1. Binding Medium

The binding medium consisted of RPMI 1640 medium without additives, 25mM N-(2-hydroxyethyl)piperazine-N'-2-ethane sulphonic acid (HEPES) and 2% BSA were prepared as 10 times concentrates in serum-free medium.

- 2%BSA: 2g of BSA is weighed and dissolved in 100ml serum free medium, aliquoted into 5ml bottles and stored at -20^{0}C.
- HEPES: 5.96g of HEPES is dissolved in 100ml serum free medium and buffered to PH 7.4 with 1M NaOH, distributed into 5ml bottles, and stored at -20^{0}C.

Serum free RPMI 1640:

Reagent	**Volume(ml)**	**Supplier**
DDH	450	-
RPMI1640 10x	50	Gibco
$NaHCO_3$, 7.5%	13.5	Sigma
NaOH, 1M	adjust to PH7.2-7.4	Alrich

Binding Buffer:

Reagents	**Volume(ml)**	**Supplier**
2% BSA	5	Sigma
5.96% HEPES	5	Sigma
Serum free 1640	40	-

2.4.2. Binding Assays with [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at Different Receptors

2.4.2.1. MC1 Receptor (B16 cells)

Cells were detached from the flasks, counted and seeded at a density of 5 X 10^5 cells per well in 24-well plates. They were then incubated for 12-16 hours before being washed twice with ice-cold RPMI 1640 serum free medium and allowed to cool to 4°C while the binding buffer was prepared. A fixed concentration of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH (normally 0.1nM) was added to the medium and different concentrations of [Nle^4, D-Phe^7]α-MSH or other non-labelled peptide were added to medium; the range was from 10^{-4} to 10^{-12}M according to peptide's affinity. 0.5ml binding medium containing [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH and the peptides were added to the wells and cells were incubated for 8hr. at 4^{0}C. After the incubation, the binding medium was washed off with ice-cold serum free medium, cells were lysed with 1M NaOH, and their radioactivity determined. The dissociation constant (Kd) was obtained using a non-linear least square regression (MINSQ) see section 2.4.5.

2.4.2.2. MC3 and MC4 Receptor (293 Cells)

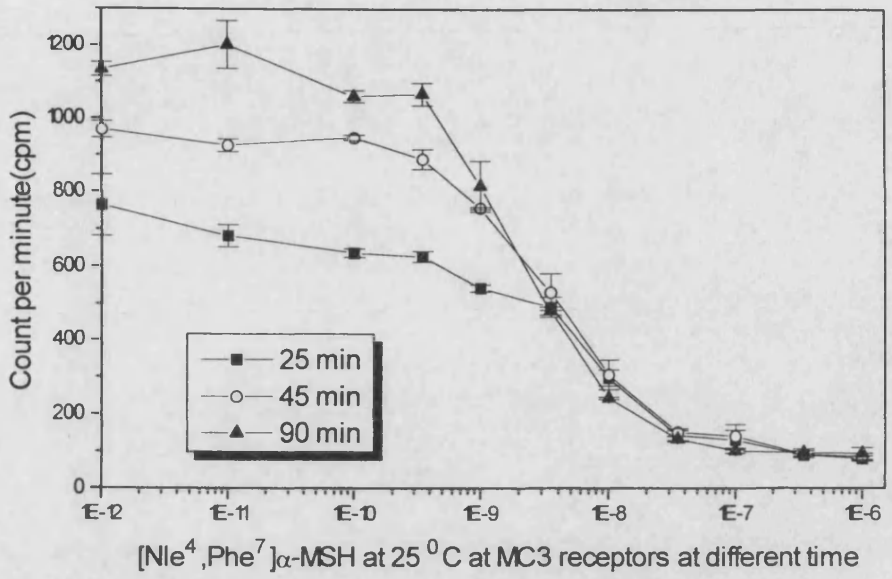
Since HEK 293 cells could not be maintained in 24-well plates at 4^{0}C for a long period, experiments were used in 96-well filtration plates. The plates were made of a solvent resistant plastic with polyvinylidene difluoride filters welded to the bottom of each plate which was removed after the incubation before counting the cell-associated radioactivity. The filter plates permitted the washing the cells to utilise a vacuum-manifold and detached cells which were not lost during the washing procedure. The plates were incubated with 10^5 cells per well and incubated for 4 hr. at 37^{0}C, then

washed three times with cold serum free medium. 0.1ml binding buffer was added and incubated for 24hr at 4^0C. After the incubation, cells was washed three times and filters with cells transferred to LP4 tubes using the Multiscreen assay system. The radioactivity was measured on the LKB 1277 Gammasmaster gamma counter.

2.4.3. Binding Assays with [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at Different Temperature and Time Intervals

The signalling of hormone and receptor is terminated by dissociation of the hormone from the receptor and/or by internalisation/inactivation of the hormone. It is not yet clear which pathway predominates in switching off a stimulated MSH and receptor complex. According to Eberle[39], association at 37^0C and 25^0C at B16 melanoma cells is rapid but does not reach a steady state, i.e. the specific binding decreases immediately after reaching its maximal value because of decomposition of the trace. At 25^0C, 50% dissociation was occurred after 90 min.

In Dr. R. Cone's laboratory, the binding assay was performed at 25^0C for 45 min. However, our binding experiment was demonstrated at 4^0C for 24 hours to reach the equilibrium binding. Hence it was attempted to compare the differences at temperature in various time in MC3 receptor at [Nle^4,D-Phe^7]α-MSH. We found that there was not much difference in the dissociation constant at 25, 45, and 90 min. compared to our result, approximately Kd=10^{-8}, but it was shown that the reaction did not reach to equilibrium at 25 and 45 minutes (or 90 min as well). This result might suggest that it require more time at 25^0C than 45 minutes.



[Nle^4,Phe^7]α-MSH at 25 ^{0}C at MC3 receptors at different time

NLDP at 25 ^{0}C				Mean	Standard Derivations
25 min	$2.86x10^{-8}$	$2.52x10^{-8}$		$2.69x10^{-8}$	$2.4077x10^{-9}$
45 min	$6.24x10^{-8}$	$1.79x10^{-8}$	$2.48x10^{-8}$	$3.50x10^{-8}$	$2.393x10^{-8}$
90 min	$3.49x10^{-8}$	$1.81x10^{-8}$		$2.65x10^{-8}$	$1.18249x10^{-8}$

2.4.4. Binding Isotherm of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

An isotherm was generated using a range of radioligand concentrations. Non-specific binding was determined using a 1000-fold excess of cold ligand. Specific binding was calculated by subtraction of total and non-specific binding.

2.4.4.1. Binding Isotherm Analysis

Receptor numbers and dissociation constants were determined using MINSQ non-linear least squares regression analysis employing the Langmuir Isotherm equation:

$$[HR] = \frac{n\,Ka\,[H]}{1+ Ka\,[H]} = \frac{n\,[H]}{Kd+ [H]}$$

Where:

[H]= Concentration of free hormone.

[HR]= Concentration of hormone-receptor complex

n= Total receptor number.

Ka= Association constant of ligand-receptor complex.

Kd= 1/Ka= dissociation constant of ligand-receptor complex.

Binding isotherms have previously been carried out in our laboratory; the affinity of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH was determined to be 0.48 nM, 1.66 nM and 7.68 nM for MC1, MC3 and MC4 respectively.

2.4.5. Analysis of Competition Binding Data

MINSQ non-linear least squares regression analysis was used to calculate dissociation constants from the competition binding data employing the following equation.

$$\mathbf{CPM}_{(test)} = CPM_{(min)} + \frac{(CPM_{(max)} - CPM_{(min)}) \times [R]}{[R] + K_{dr} \times \frac{[I]}{K_i}}$$

Where:

$CPM_{(test)}$= CPM Associated with a given competitor concentration

$CPM_{(max)}$= CPM Associated with maximum radioligand binding without competitor.

CPM $_{(min)}$= CPM Associated with minimum radioligand binding with excess competitor.

[R]= Concentration of radiotracer

[I]= Concentration of competitor

K_{dr}= Dissociation constant of radiotracer

K_i= Dissociation constant of competitor

2.5. cAMP Accumulation

Biological activity of the test peptides was analysed by measurement of cAMP concentration after stimulation of the receptor. The method was based on the procedure described by Salomon et al.[181,182].

2.5.1. Solutions

- 2% Bovine serum albumin (BSA) (Sigma): 2g of BSA were weighted and dissolved in 100ml serum free medium as stock solution and stored at -20^{0}C.
- 10mM 3-isobutyl-1-methylxanthine; IBMX (Sigma): 22.23 mg were dissolved in 10 ml DD water in a boiling water bath as a stock solution and stored at -20^{0}C for a maximum of three months.
- 25% v/v Perchlorate; PCA (BDH Chemicals) was prepared as a stock solution and kept in the dark at room temperature.

- 0.1 M cAMP (Sigma): 351.2 mg were weighed in 10 ml DD water as stock solution and stored at -20^{0}C.
- 4.2 N KOH (BDH Chemicals) 117.56g were dissolved to 500 ml DD water as a stock solution and kept at room temperature.
- Preincubation medium: contained *[8-^{3}H]*Adenine (Amersham) 0.025µCi/ well (0.05µCi/ml) in RPMI 1640 culture medium.
- Incubation medium: contained 0.1 mg/ml BSA and 0.1 mM IBMX in serum-free medium as well as various concentrations of peptide.
- Stop solution: PCA 2.5%, cAMP 0.1 mM were prepared freshly from stock solution and used ice cold.

2.5.2. Assay Procedure

Cells were seeded into 24-well plates at a density of 5 x 10^5 cells /well and incubated overnight at 37^{0}C. in a humidified atmosphere of 5 % CO_2 and 95% air. Different cell lines required different incubation times for attachment, as B16 needed around 16 hours however, MC3 took up to 22 hours. Cells were incubated with prewarmed preincubation medium for 2 hours at 37^{0}C. The radioactive medium was removed and the cells washed with 0.5ml prewarmed PBS. Serial dilutions of peptides were made in the incubation medium. Cells were stimulated with 0.5 ml samples of this medium for 45 minutes at 37^{0}C. Each concentration was assayed in triplicate. The medium was removed and stop solution added to each well for 30 minutes at 4^{0}C. The culture dishes were occasionally agitated for efficient [^{3}H]-cAMP extraction. 0.8 ml PCA

extracts were transferred to clean tubes and neutralised with 80 µl 4.2N KOH. A sediment of potassium perchlorate could be observed. Samples could be store at 4^0C overnight or at -20^0C for longer periods[181].

Distilled water was added to each tube to a final volume of 1.3 ml and mixed to allow crystals to resettle. A 0.9 ml sample was submitted to double-column chromatography in order to separate the *[^{3}H]*cAMP from [^{3}*H*]cATP and other radioactive contaminants[183].

2.5.3. Column Apparatus and Reagents

2.5.3.1. Columns

Two sets of 17 ml polypropylene separation columns (Mitchell Plastics Inc.) with plastic filter discs were mounted over each other in a tiered rack set which in turn was suspended over 4 ml collection tubes.

2.5.3.2. Reagents

- Dowex 50 AG50W-X8 resin 100-200 mesh (Bio-Rad): before use the Dowex 50 was washed with 6 volumes each of 0.1N NaOH, water, 1N HCl and water. and poured into the first set of columns in an approximately 2:1 slurry. After each use, Dowex 50 columns were regenerated by washing with 5 ml of 1N HCl, and stored

until reused. Before use the columns were washed 3 times with 10 ml of water. The columns could be reuse several times.

- Neutral aluminated 100-200 mesh (Sigma WN-3): amunima 0.6 g was poured dry into the lower column set. Before initial use alumina columns must be washed once with 10 ml of 100 mM Tris-Cl at pH 7.5. After each use the columns are washed with 10 ml of 100 mM Tris-Cl at pH 7.5. The columns could be reused several times.
- Elution buffers was 100mM Tris-Cl(Sigma), pH 7.5. The purpose of the buffer was to elute cyclic nucleotides. Since eluate from the Dowex 50 columns was acidic, which enhanced adsorption of cyclic nucleotides to alumina, elution of cyclic nucleotides was achieved through an increase in the pH of the buffer.
- 1N HCl was prepared for final washed of Dowex 50.

2.5.4. Column Chromatography Procedure

The Dowex 50 columns were washed with 30ml distilled water and the alumina columns with 8 ml 100 mM Tris-Cl, which the PH is 7.5., before samples were added. The neutralised PCA extract solutions 0.9 ml were pipetted into Dowex 50 columns, leaving the perchlorate crystals undisturbed. Each column was washed with 3 ml water and the eluate discarded. The Dowex 50 columns were mounted above an equal number of the alumina columns and washed with 8 ml water. The eluate from the Dowex 50 columns was slightly acidic and caused retention of *[^{3}H]*cAMP was on the alumina. Then columns were placed over collection tubes and *[^{3}H]*cAMP was eluted

with 4 ml 100mM Tris-Cl. A 0.5 ml sample of each eluate was added to 4 ml Optiphase scintillation cocktail (Wallac) and vigorously mixed. Radioactivity of the scintillation vial contents was quantified on a LKB Wallac 1215 RackBeta liquid scintillation counter. The Dowex 50 columns were regenerated after each use with 5 ml 1N HCl and alumina columns with 8 ml Tris-Cl.

2.5.5. Calculation of EC_{50} values

MINSQ non-linear least squares regression analysis was used to calculate EC_{50} values for the tested peptides employing the following equation:

$$DPM_{(test)} = DPM_{(max)} + \frac{(DPM_{(min)} - DPM_{(max)})}{1 + \frac{[C]}{EC_{50}}}$$

Where:

$DPM_{(test)}$= DPM Associated with a given peptide concentration

$DPM_{(max)}$= DPM Associated with maximum stimulation

$DPM_{(min)}$= Background dpm without stimulation

[C]= Peptide concentration

EC_{50}= Concentration required to produce half maximal stimulation

2.5.6. Determination of Antagonist Activity

Cells of 5 x 10^5 cells/well were plated in 24-well tissue culture dishes and grown in RPMI1640 medium at 37 °C for overnight. Cells were incubated for 2 hours with

prewarmed preincubation medium at 37^0C. Medium was aspirated and the cells were washed once with prewarmed PBS. The cells were then exposed for 45 min at 37^0C to varying concentrations of peptides in the presence of serum-free medium containing 0.1% BSA, 0.5 mM IBMX and 10^{-8} M α-MSH. The medium with peptides was aspirated off and the cells solubilized with stop solution, which was 1 ml 2.5 % perchloric acid, 0.1mM cAMP for 30 min at 4^0C. Lysate 0.8 ml was removed, neutralised with 80µl 4.2 NKOH, and 0.42 ml H_2O. The samples were mixed and the sediment was allowed to settle. [^3H]-cAMP was separated from the lysate after sequential chromatography over Dowex and alumina columns as described in section 2.5.4 .

2.5.7. Calculation of IC_{50} Values

$$DPM = DPM_{(min)} + \frac{(DPM_{(max)} - DPM_{(min)}) \times [C_{\alpha MSH}]}{[C_{\alpha MSH}] + EC_{50\alpha MSH} \times \frac{[C]}{IC_{50}}}$$

Where $DPM_{(min)}$ = Background DPM

$DPM_{(max)}$ = Maximum counts

$C_{\alpha\text{-}MSH}$ =Fixed concentration of α-MSH

$EC_{50\alpha\text{-}MSH}$ = EC_{50} of α-MSH in control experiment

C = Concentration of antagonist

IC_{50} = IC_{50} of antagonist

IC_{50} values were calculated with the above equation using MINSQ non-linear least square regression

2.6. Statistical Analysis

Data analysis was performed using MINITAB 9.1. Significant differences were determined using a one-way analysis of variance following Fisher's multiple comparison procedure at the 95% confidence interval.

Chapter 3: Activity of C-terminally Modified Melanocortin Analogues at MC1 and MC3 Receptors

3.1. Introduction

The results presented in this chapter are concerned with the putative differences in activation between the MC1-R and the MC3-R. The design of selective ligands for either subtype of the receptor would greatly enhance the possibilities for studying receptor subtype function and might also have clinical prospects.

Structure-activity studies with natural and synthetic analogues have been carried out for all the receptors known to date, but the analogues tested show only a small degree of selectivity towards one or the other of the receptor subtypes. A common feature of all melanocortin receptors is that the synthetic analogue [Nle^4,D-Phe^7]α-MSH[23] appears to be more potent than the endogenous ligand, α-MSH. However, on the MC3-R isolated from rat hypothalamus, most known naturally occurring MSH peptides act with the same potency[134], implying that the rat MC3-R is more sensitive to γ-MSH than are other melanocortin receptors.

α-MSH and γ-MSH show structural differences mainly within the C-terminus and it has already been suggested that the presence of proline 12 (number with reference to α-MSH) is an important feature for the binding of α-MSH to the MC1-R[134], but not the MC3-R[135,184]. Eberle, however, suggested that position 11 is very important for the activity of the C-terminus of α-MSH at MC1-R, for since Trp-Gly-OH and H-Pro-Val-NH_2 are inactive, it appears that Lys^{11} is the crucial position within the C-terminus[39]. However, when Sahm *et al.*[134] replaced every

position of α-MSH by alanine, there were some degrees of difference observed at position Pro^{12} between the MC1-R and MC3-R rather than at position 11. Cody *et al.*[142] demonstrated that Ac-[Cys^4,Cys^{10}]α-MSH_{4-12}-NH_2 is more potent than Ac-[Cys^4,Cys^{10}] α-MSH_{4-11}-NH_2 and is equipotent as [Cys^4,Cys^{10}] α-MSH at the frog, lizard and mouse melanoma. Sawyer *et al.*[143] also pointed out that Lys^{11} in Ac-α-MSH_{6-11}-NH_2, was not essential to the potency of this α-MSH fragment derivative for it only showed a relative potency of 5.0 in both the frog and lizard skin compared to Ac-α-MSH_{6-9}-NH_2. However, addition of Pro^{12} to Ac-α-MSH_{6-12}-NH_2 exhibited markedly improved potency relative to Ac-α-MSH_{6-9}-NH_2 in both bioassays (500-fold in frog skin and 100-fold in lizard skin). Therefore, Pro^{12} significantly contributed to melanotropic potency[143].

To test this hypothesis C-terminally modified analogues of both α-MSH and γ-MSH were synthesised to investigate whether the C-terminus plays a role in promoting selectivity of melanocortin peptides. These analogues were tested for receptor binding and stimulation of adenylyl cyclase in B16 murine melanoma cells expressing the MC1-R and HEK-293 cells stably transformed to express the gene encoding the rat hypothalamus MC3-R.

3.2. Results

All peptides tested were able to inhibit completely the binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to both receptors and exhibit full biological activity at appropriate concentrations (Table.3.1, Table.3.2 and Table.3.4). To facilitate the comparison of affinity and activity at both receptors, the data is presented relative to α-MSH in Table. 3.3 and Table 3.5.

3.2.1. C-Terminal Modified α-MSH and γ-MSH Analogues

3.2.1.1. MC1 Receptor

Data from representative experiments are shown in Fig. 3.1 and 3.2. At the MC1-R, all compounds derived from [Nle^4,D-Phe^7]α-MSH, a potent synthetic analogue of α-MSH[181], showed approximately 10 times higher affinity and activity than α-MSH itself (compounds **2-5**) (Table.3.1and 3.3A). Among the substitutions, [Nle^4,D-Phe^7,Phe^{12}]α-MSH had slightly higher affinity than the other compounds **2-5** (Fig.3.1.A). [Nle^4,D-Phe^7]α-MSH and [Nle^4,D-Phe^7,Leu^{12}]α-MSH had similar affinity to each other. [Nle^4,D-Phe^7]α-MSH had slightly higher activity than other compounds although compounds **3-5** were not significantly different (Fig 3.1B and Table 3.1). Neither affinity nor activity were affected by substitutions in position 12 (Fig.3.1A and 3.1B). However, when α-MSH itself was substituted with phenylalanine in position 12, the potency of the resulting peptide (compound **7**) (Table.3.1) was reduced to 1-2 % of that of the natural ligands potency (Fig.3.2). Substitution in position 10 with aspartate showed a similar effect. When both substitutions were made (compound **8**) the peptide was rendered virtually inactive (Table 3.3A) with 0.06% of the affinity and 0.009% of the activity of α-MSH.

γ_1-MSH (compound **9**) showed very low affinity and activity at the MC1-R (0.4% and 0.06% that of α-MSH, respectively). Its synthetic analogue [Nle^3]γ_1-MSH (compound **10**), was synthesised in analogy to [Nle^4, D-Phe^7]α-MSH, and on the basis that this peptide would be more resistant to oxidation. It was decided not to

introduce the D-Phe residue in position 7 as this had proven to encourage high affinity binding to the receptor regardless of amino acid replacements within the C-terminal region when substituting α-MSH analogues in this study. Several substitutions were made in position 11 (corresponding to position 12 in α-MSH). Most of these analogues (compound **9-14**) (Table.3.1) exhibited approximately the same activity as γ_1-MSH itself. For [Nle^3]γ_1-MSH the biological activity could not be determined; it appeared to be very low ($EC_{50} > 10^{-6}M$), but results were inconclusive. However, an analogue that was substituted with proline in position 11, [Nle^3,Pro^{11}]γ_1-MSH (compound **12**), showed significantly increased binding and biological activity at the MC1-R when compared to γ_1-MSH (Table 3.1). In terms of binding affinity, [Nle^3, Pro^{11}]γ_1-MSH shows the similar to [Phe^{12}]α-MSH and in biological activity, [Nle^3, Pro^{11}]γ_1-MSH demonstrated similar activity to [Asp^{10}]α-MSH. Nevertheless, even this peptide did not show more than 2-3% of the affinity and activity of α-MSH (Table 3.3A).

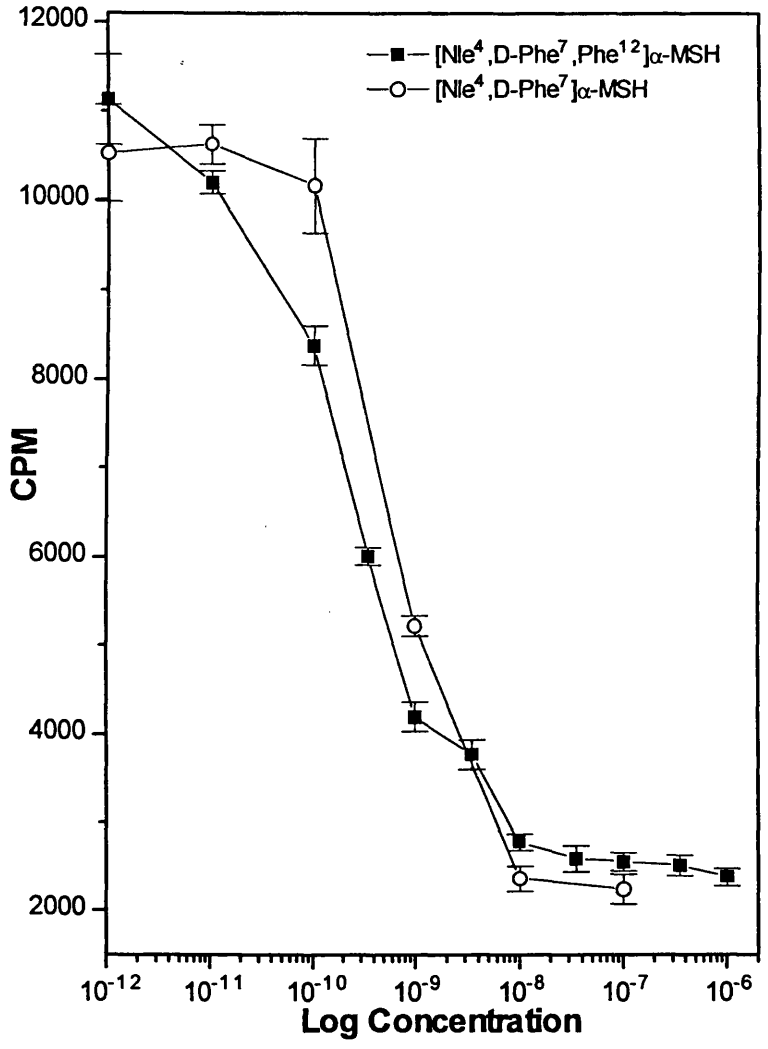


Figure.3.1A: Displacement of [^{125}I-Tyr^2, Nle^4, D-Phe^7]α-MSH from the MC1-R by [Nle^4, D-Phe^7,Phe^{12}]α-MSH and [Nle^4,D-Phe^7]α-MSH.

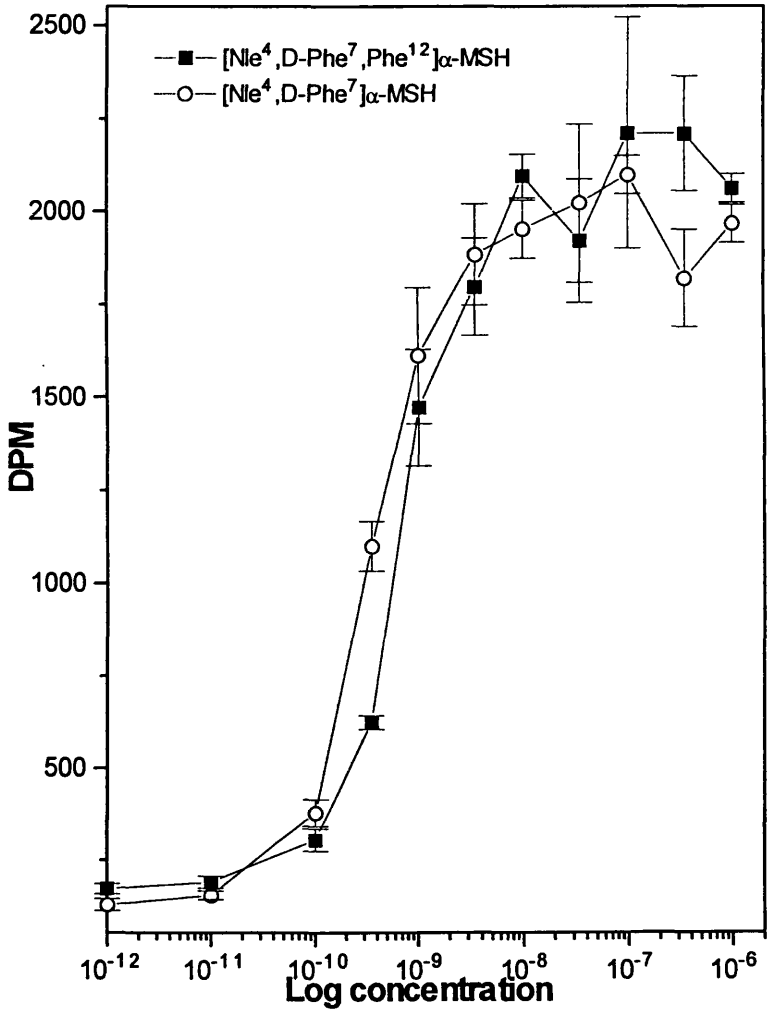


Figure.3.1B: Stimulation of cAMP production at MC1R by [Nle^4, D-Phe^7, Phe^{12}]α-MSH and [Nle^4, D-Phe^7]α-MSH.

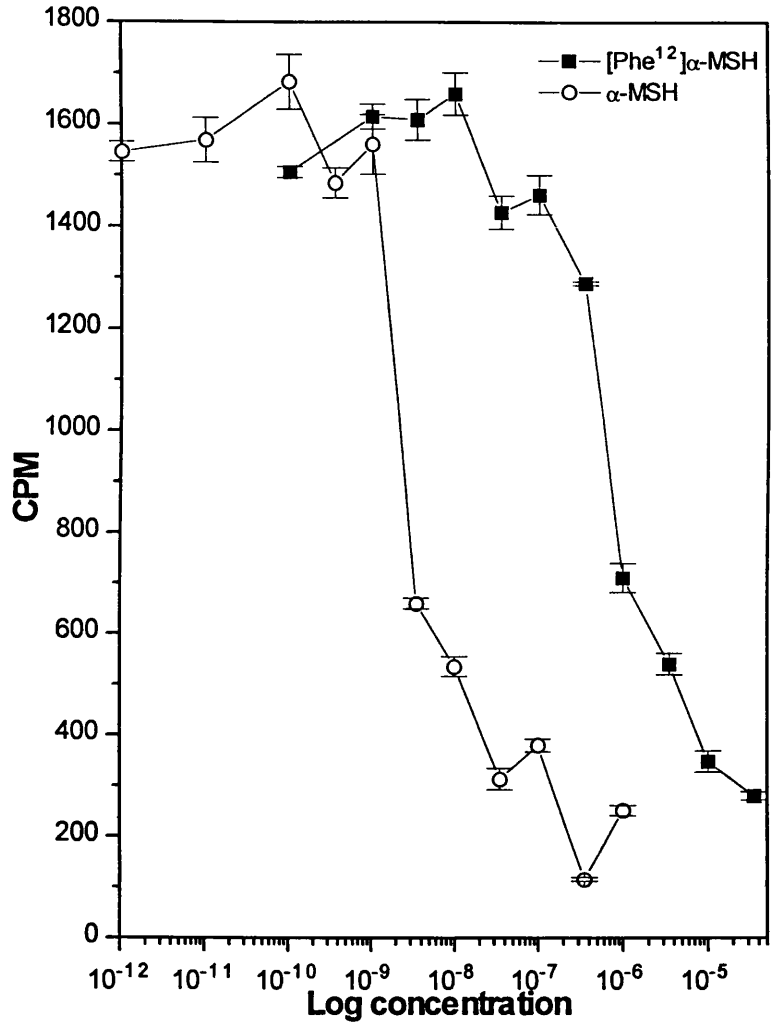


Figure.3.2A: Displacement of [^{125}I-Tyr^{2}, Nle^{4}, D-Phe^{7}]α-MSH from the MC1-R by [Phe^{12}]α-MSH and α-MSH.

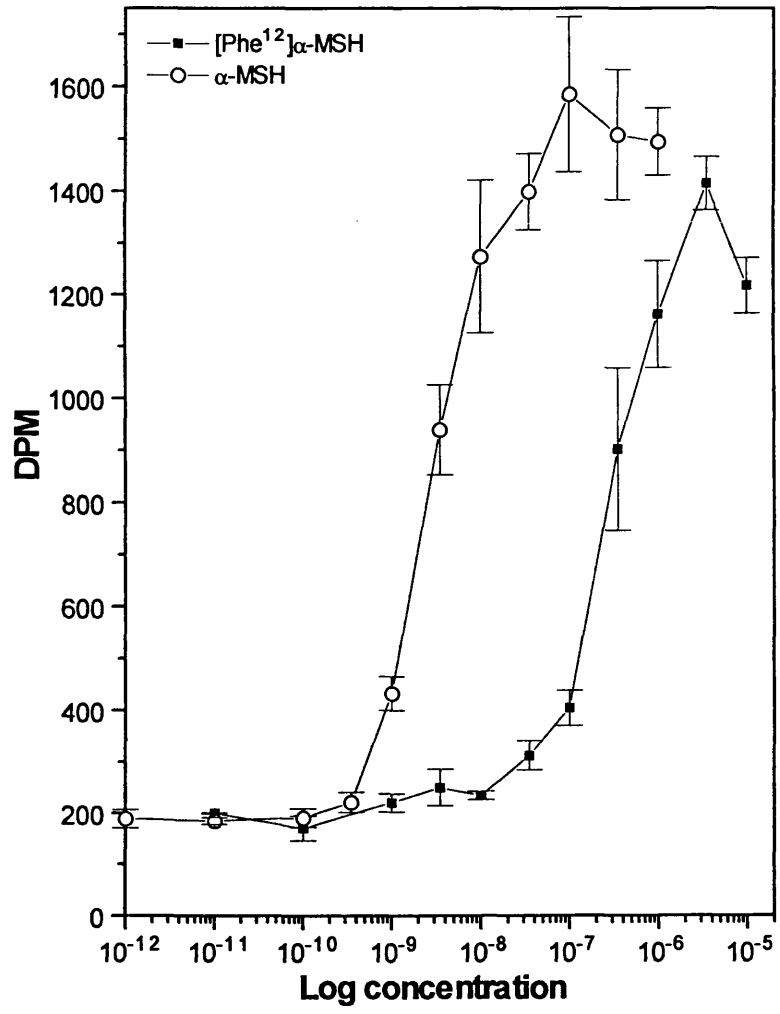


Figure.3.2B: Stimulation of cAMP production at MC1R by [Phe^{12}]α-MSH and α-MSH.

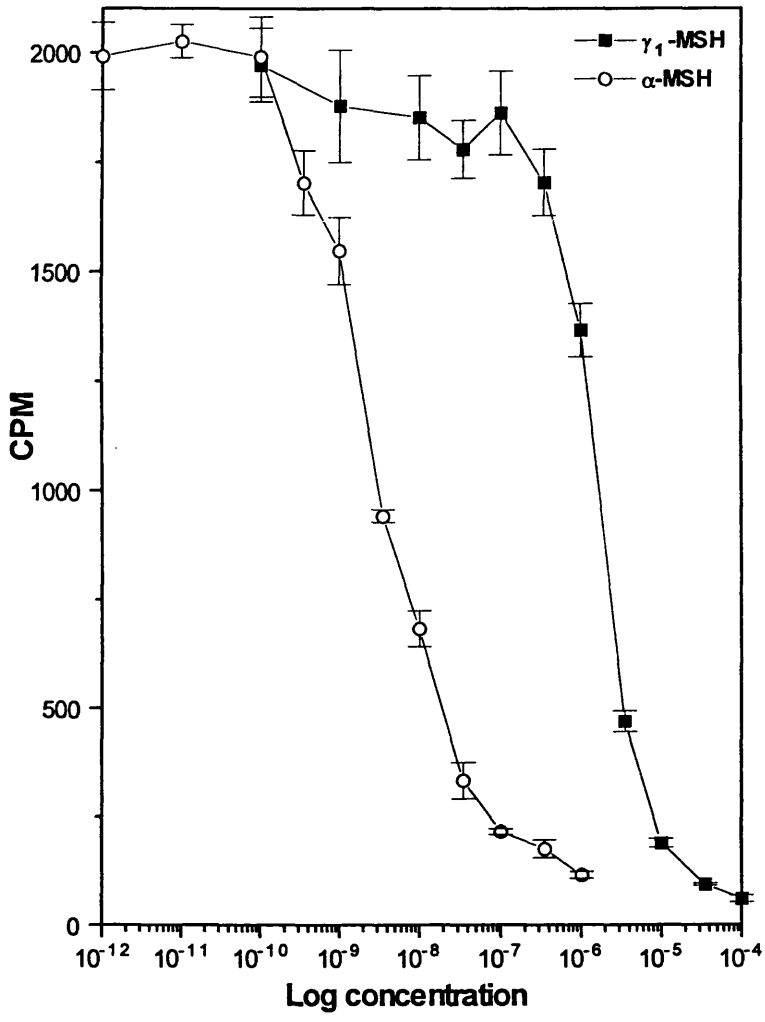


Figure 3.2.C. Displacement of [^{125}I-Tyr^2, Nle^4, D-Phe^7]α-MSH from the MC1-R by α-MSH and γ_1MSH.

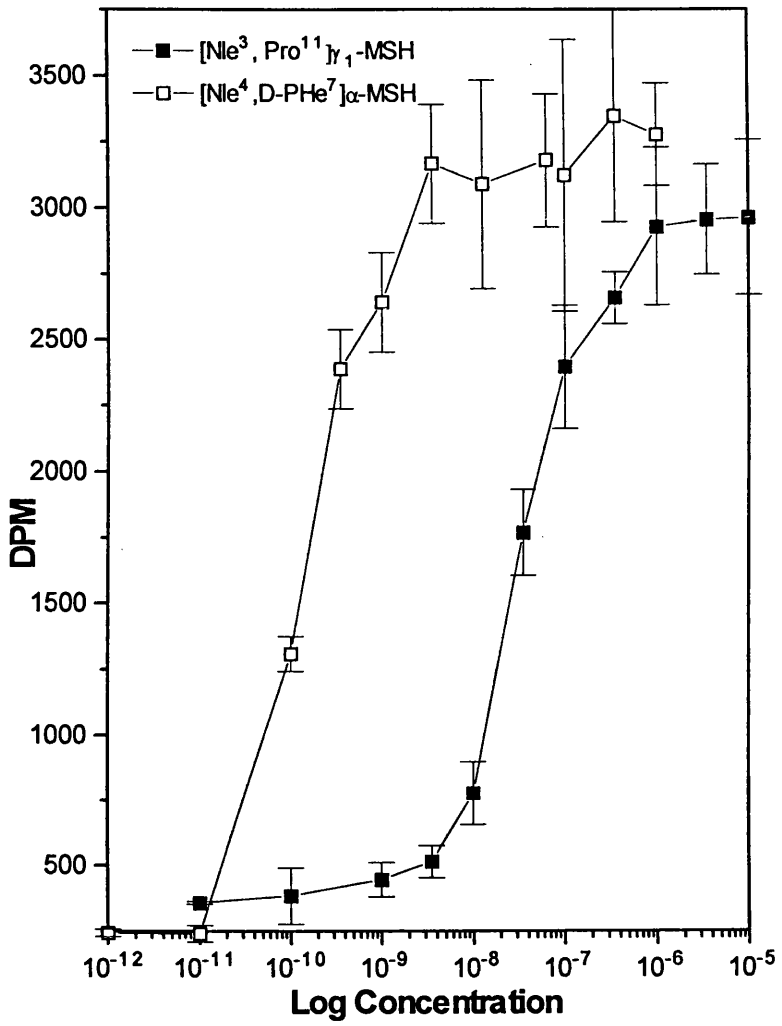


Figure 3.2.D. Stimulation of cAMP production at MC1R by [Nle^3,Pro^{11}]α-MSH and [Nle^4,D-Phe^7]α-MSH.

Table.3.1. Molar dissociation constants, EC_{50} values, and their standard deviations for α-MSH analogues in B16 melanoma cells. Each value is the mean of three or more experiments (Statistics data, appendix 1.1 & 1.2).

	No	Binding (K_d)	c-AMP (EC_{50})
α-MSH	1	1.68×10^{-8} $\pm 3.88 \times 10^{-9}$ n=5	2.74×10^{-9} $\pm 9.56 \times 10^{-10}$ n=6
[Nle^4,D-Phe^7]α-MSH	2	3.27×10^{-9} $\pm 1.78 \times 10^{-9}$ n=5	1.67×10^{-10} $\pm 1.30 \times 10^{-10}$ n=6
[Nle4,D-Phe^7,Phe^{12}]α–MSH	3	9.80×10^{-10} $\pm 9.97 \times 10^{-11}$ n=3	5.93×10^{-10} $\pm 2.58 \times 10^{-10}$ n=5
[Nle^4,D-Phe^7,Ser^{12}]α-MSH	4	1.88×10^{-9} $\pm 8.33 \times 10^{-10}$ n=3	3.08×10^{-10} $\pm 1.23 \times^{-10}$ n=3
[Nle^4,D-Phe^7,Leu^{12}]α-MSH	5	2.70×10^{-9} $\pm 7.42 \times 10^{-10}$ n=5	2.38×10^{-10} $\pm 1.33 \times 10^{-10}$ n=4
[Asp^{10}]α-MSH	6	1.39×10^{-6} $\pm 6.13 \times 10^{-7}$ n=3	2.25×10^{-7} $\pm 2.06 \times 10^{-7}$ n=3
[Phe^{12}]α-MSH	7	5.25×10^{-7} $\pm 3.78 \times 10^{-7}$ n=3	4.50×10^{-7} $\pm 1.61 \times 10^{-7}$ n=3
[Asp^{10},Phe^{12}]α-MSH	8	2.74×10^{-5} $\pm 3.2 \times 10^{-5}$ n=3	3.06×10^{-5} $\pm 2.35 \times 10^{-5}$ n=3
γ_1-MSH	9	3.86×10^{-6} $\pm 2.31 \times 10^{-6}$ n=3	4.47×10^{-6} $\pm 1.53 \times 10^{-7}$ n=3
[Nle^3]γ_1-MSH	10	1.35×10^{-5} $\pm 7.62 \times 10^{-6}$ n=3	$>>1.0 \times 10^{-6}$ n=3
N$^\alpha$-Lys-[Nle^3]γ_1-MSH	11	1.96×10^{-5} $\pm 8.45 \times 10^{-6}$ n=3	6.00×10^{-6} $\pm 2.83 \times 10^{-6}$ n=2
[Nle^3,Pro^{11}]γ_1-MSH	12	5.82×10^{-7} $\pm 4.61 \times 10^{-7}$ n=3	1.36×10^{-7} $\pm 1.11 \times 10^{-7}$ n=4
[Nle^3,Ser^{11}]γ_1-MSH	13	7.28×10^{-6} $\pm 3.11 \times 10^{-6}$ n=3	2.97×10^{-6} $\pm 1.16 \times 10^{-6}$ n=3
[Nle^3,Leu^{11}]γ_1-MSH	14	1.53×10^{-5} $\pm 3.80 \times 10^{-6}$ n=3	3.73×10^{-6} $\pm 2.85 \times 10^{-6}$ n=3

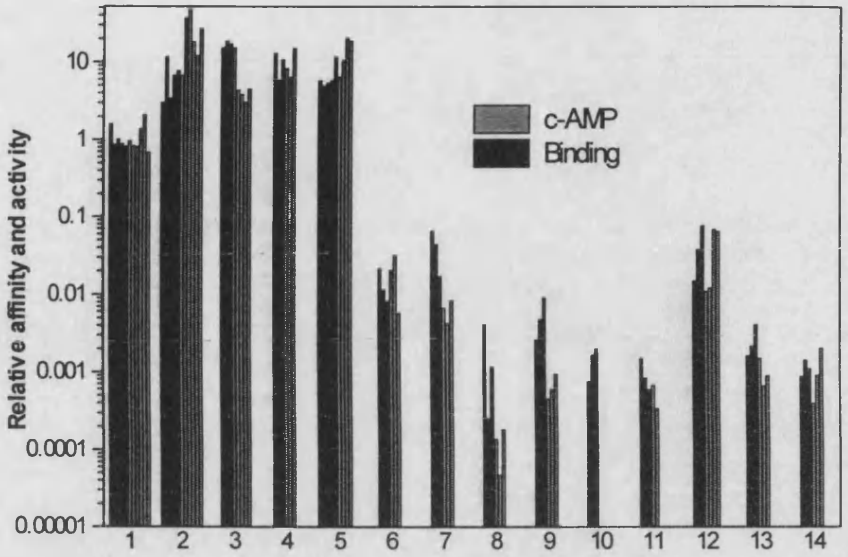


Figure.3.7A : Relative affinity and activity of α-MSH and γ_1-MSH analogues to α-MSH at MC1-R (log scale). Individual bars represent single experiments. Black = Affinity, Grey = Activity.

	No	Binding(K_d)	c-AMP(EC_{50})
α-MSH	**1**	1.0	1.0
[Nle^4,D-Phe^7]α-MSH	**2**	5.14	16.4
[Nle4,D-Phe^7,Phe^{12}]α–MSH	**3**	17.14	4.62
[Nle^4,D-Phe^7,Ser^{12}]α-MSH	**4**	8.94	8.9
[Nle^4,D-Phe^7,Leu^{12}]α-MSH	**5**	6.2	11.5
[Asp^{10}]α-MSH	**6**	0.012	0.012
[Phe^{12}]α-MSH	**7**	0.032	0.006
[Asp^{10},Phe^{12}]α-MSH	**8**	0.0006	0.00009
γ_1-MSH	**9**	0.004	0.0006
[Nle^3]γ_1-MSH	**10**	0.0012	n.d.
N$^{\alpha}$-Lys-[Nle^3]γ_1-MSH	**11**	0.0009	0.0005
[Nle^3,Pro^{11}]γ_1-MSH	**12**	0.029	0.02
[Nle^3,Ser^{11}]γ_1-MSH	**13**	0.002	0.0009
[Nle^3,Leu^{11}]γ_1-MSH	**14**	0.001	0.0007

Table.3.3A : Relative affinity constants and EC_{50} for α-MSH analogues in B16 melanoma cells.

3.2.1.2. MC3 Receptor

At the MC3-R, the binding affinities of [Nle^4,D-Phe^7]α-MSH and its analogues **2-5** (Table 3.2 and Table 3.3B) were 30-80-fold stronger than that of α-MSH; their biological activities were higher by a factor of 5-20 (Fig. 3.3B). Among the compounds **2-5**, they were not significantly different in affinity and activity whereas [Nle^4,D-Phe^7,Ser^{12}]α-MSH showed higher affinity and [Nle^4,D-Phe^7]α-MSH had slightly higher activity than other analogues. Substitution in position 12 in compounds **2-5** did not appear to make a significant contribution to affinity or activity. However, when substituting phenylalanine for proline in position 12 of α-MSH itself (compound **7**), the affinity of the peptide was reduced to 10%, whereas the activity remained unchanged compared to α-MSH (Fig.3.4). The same effect was observed when position 10 was changed from glycine to aspartate (**6**); the affinity was affected more markedly (reduction to 0.8%) than the biological activity of the peptide (reduction to 20% that of α-MSH). The [Asp^{10},Phe^{12}]α-MSH analogue showed the same properties as [Asp^{10}]α-MSH (Fig.3.4A).

None of the γ_1-MSH peptides compounds **9-14** (Table.3.2) exhibited a very high affinity for the rat MC3-R. Their dissociation constants were all in the micromolar range and their affinities did not exceed 5% of that of α-MSH. Among compounds **9-14**, the [Nle^3,Pro^{11}]γ_1-MSH and [Nle^3]γ_1-MSH had slightly higher affinity than other γ_1-MSH analogues. However, when compared to the MC1-R, their biological activity appeared to be significantly less reduced (Table.3.1 and Table.3.2) than that of α-MSH. [Nle^3,Pro^{11}]γ_1-MSH had similar activity to γ_1-MSH and still higher than other compounds (**9-14**). [Nle^3]γ_1-MSH, N$^{\alpha}$-Lys-[Nle^3]γ_1-MSH, and

[Nle^3,Leu^{11}]γ_1-MSH had lower activity among γ_1-MSH analogues although their affinities were similar to γ_1-MSH analogues.

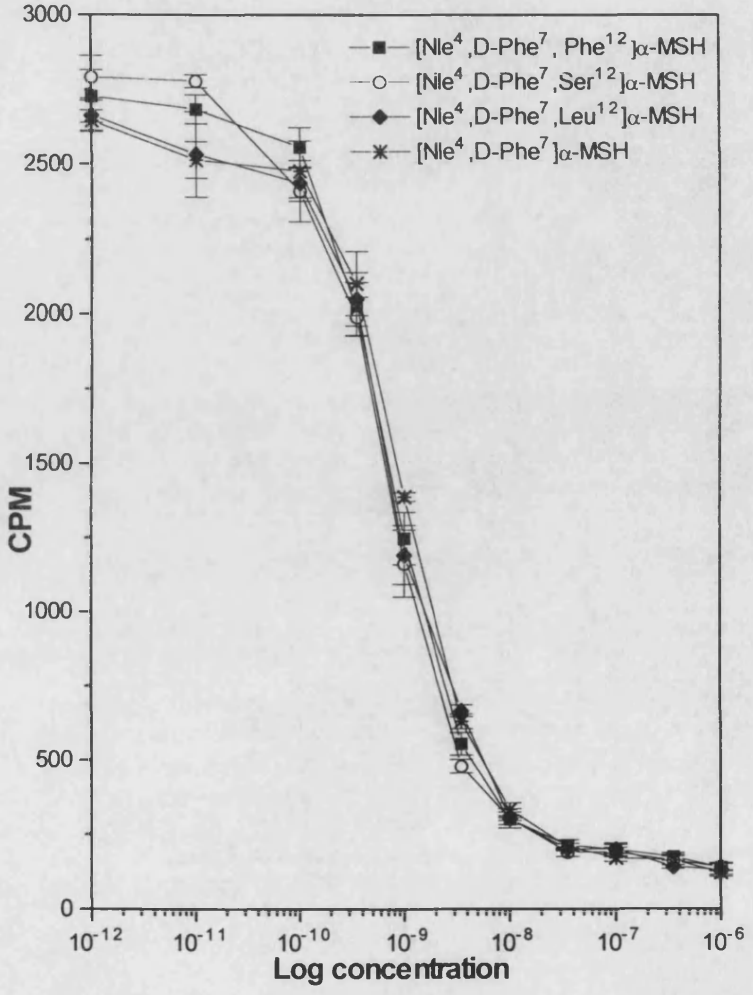


Figure.3.3A: Displacement of [^{125}I-Tyr^2, Nle^4, D-Phe^7]α-MSH from the MC3-R by [Nle^4, D-Phe^7] α-MSH analogues.

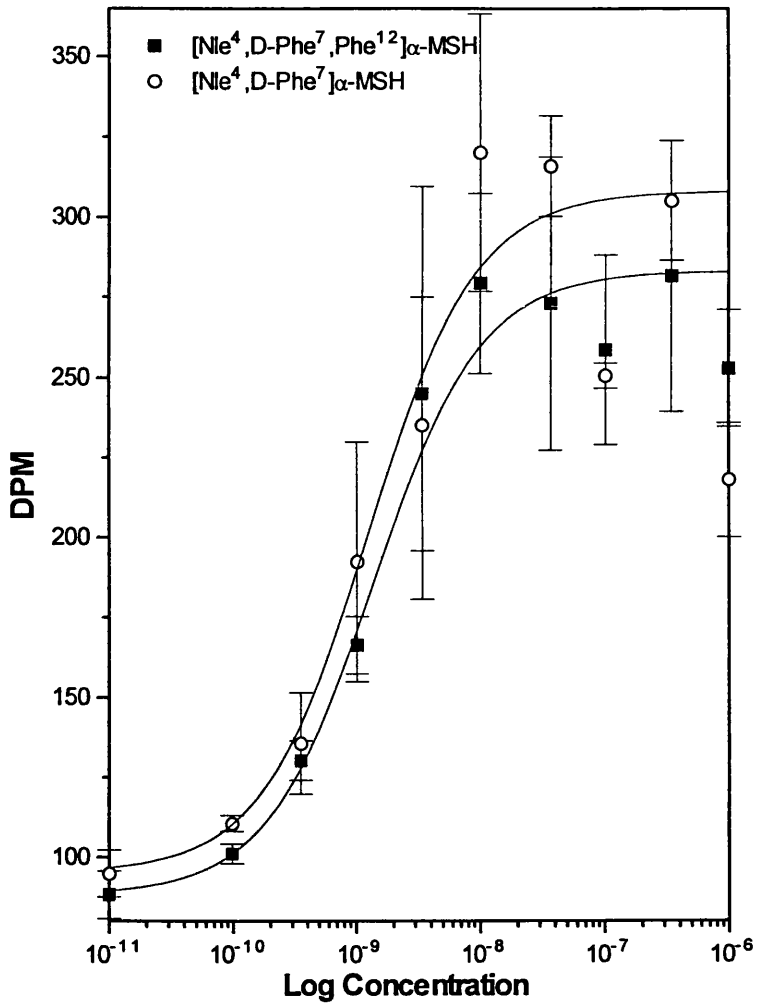


Figure.3.3B : Stimulation of cAMP production at MC3-R by [Nle^4, D-Phe^7]α-MSH and [Nle^4, D-Phe^7, Phe^{12}]α-MSH.

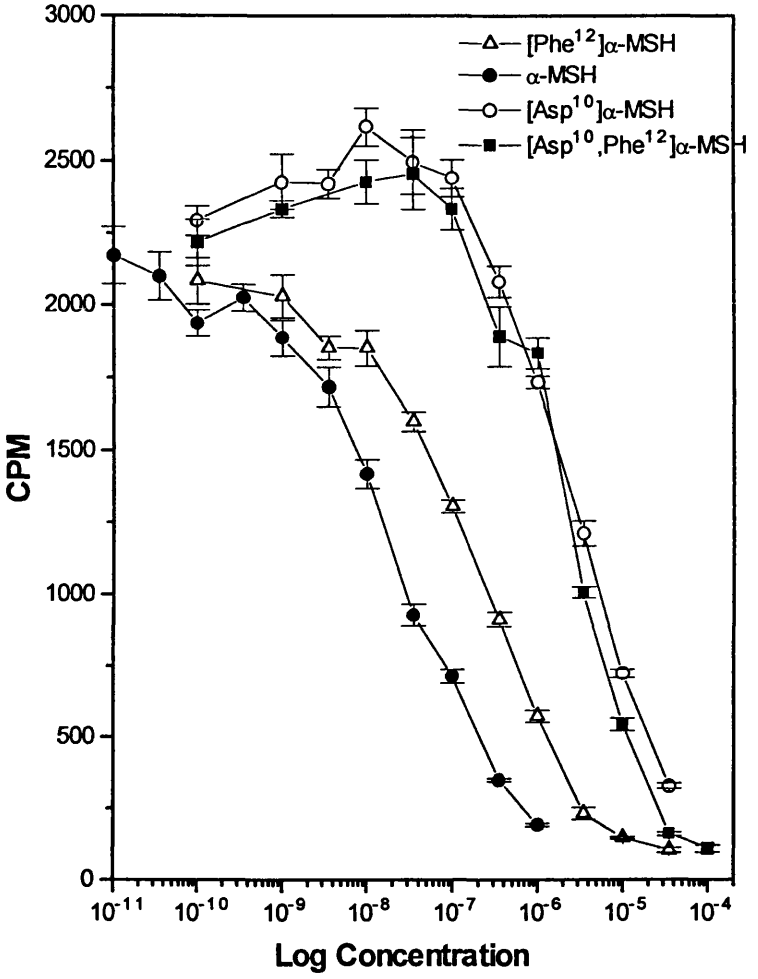


Figure.3.4A : Displacement of [^{125}I-Tyr^2, Nle^4, D-Phe^7]α-MSH from the MC3-R by α-MSH and α-MSH analogues.

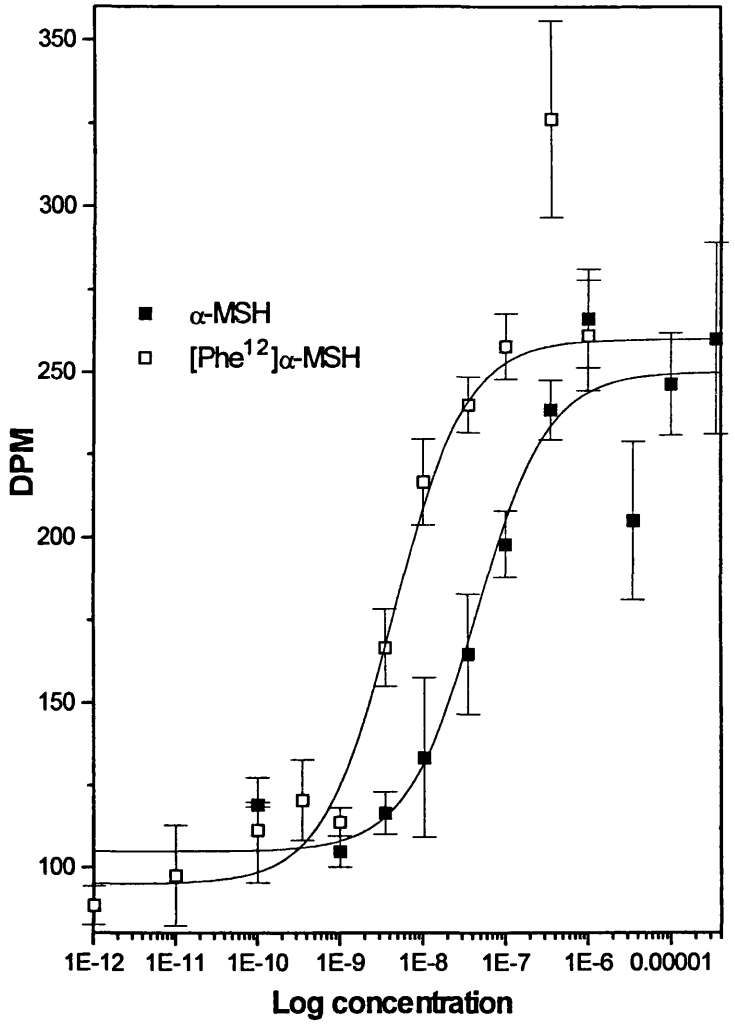


Figure.3.4B : Stimulation of cAMP production at MC3-R by [Phe^{12}]α-MSH and α-MSH.

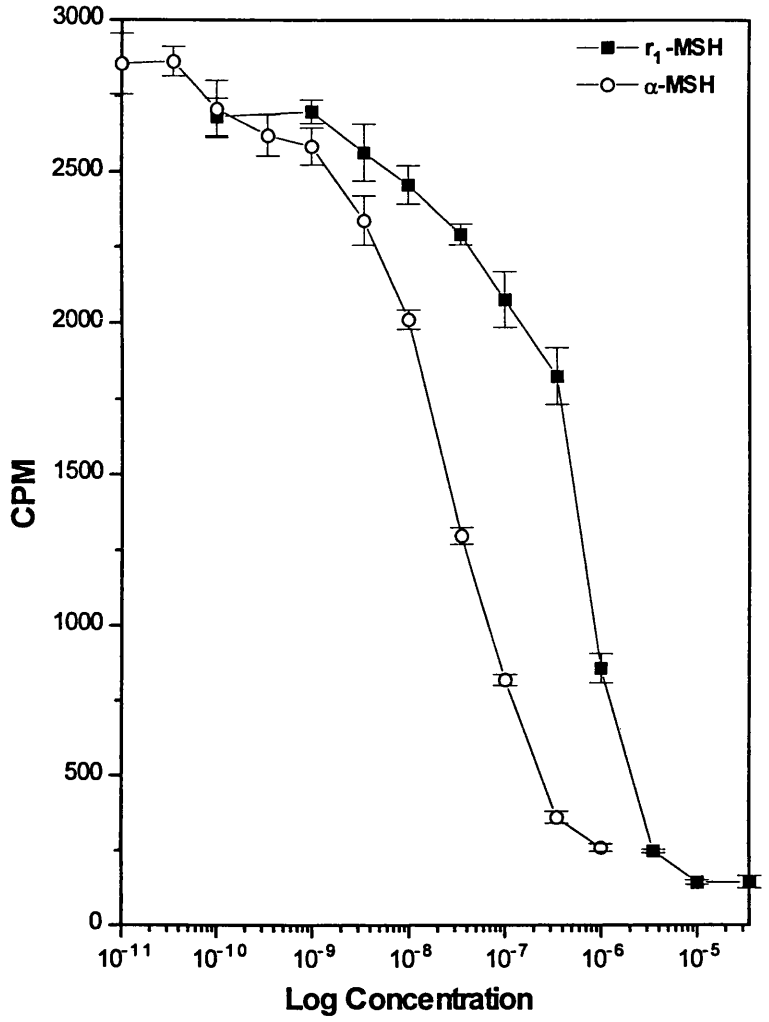


Figure 3.4.C. Displacement of [^{125}I-Tyr^2, Nle^4, D-Phe^7]α-MSH from the MC3-R by α-MSH and γ_1-MSH.

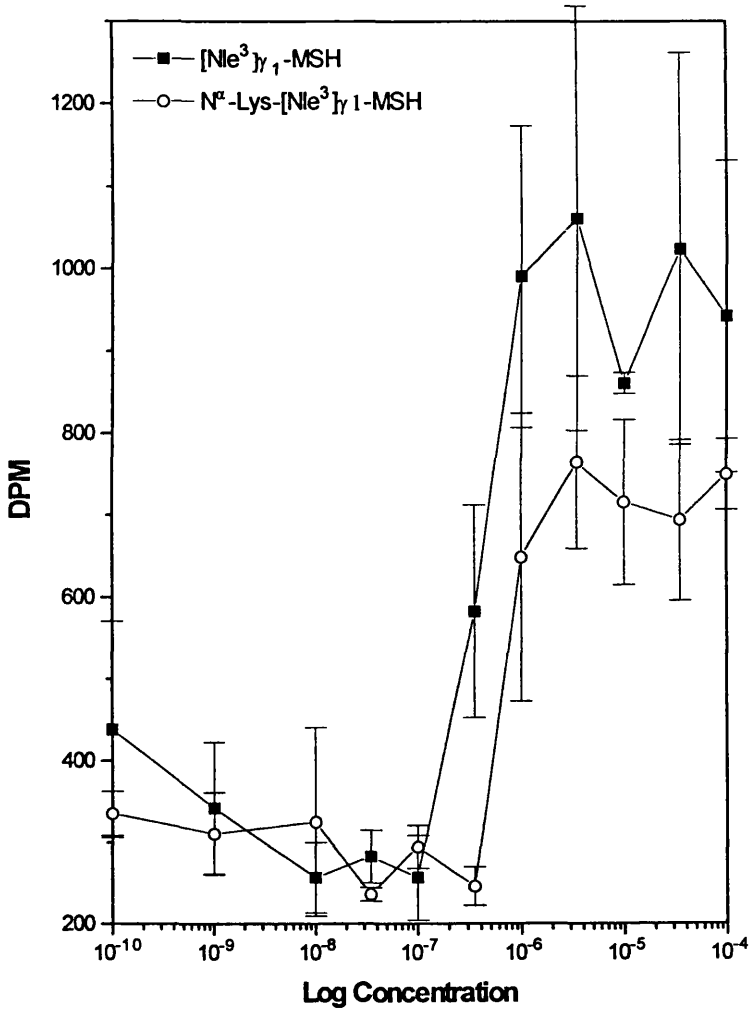


Figure 3.4.D. Stimulation of cAMP production at MC3-R by γ_1-MSH and α-MSH.

Table.3.2. Molar dissociation constants, EC_{50} values, and their standard deviation for α-MSH analogues at MC3-R cell. n= The number of the experiments (Statistics data, appendix 1.3 & 1.4).

	No	Binding(K_d)	c-AMP(EC_{50})
α-MSH	1	6.59×10^{-7}± 6.60 3.14×10^{-7} n=3	2.36×10^{-8} ± 2.55×10^{-8} n=4
[Nle^4,D-Phe^7]α-MSH	2	2.16×10^{-8} ± 1.18×10^{-8} n=5	1.25×10^{-9} ±8.20×10^{-10} n=3
[Nle4,D-Phe^7,Phe^{12}]α–MSH	3	1.22×10^{-8} ± 4.85×10^{-9} n=5	3.58×10^{-9} ± 1.96×10^{-9} n=3
[Nle^4,D-Phe^7,Ser^{12}]α-MSH	4	8.21×10^{-9} ± 2.49×10^{-9} n=5	2.73×10^{-9} ±1.58×10^{-9} n=3
[Nle^4,D-Phe^7,Leu^{12}]α-MSH	5	1.62×10^{-8} ± 6.91×10^{-9} n=6	2.89×10^{-9} ± 1.59×10^{-9} n=3
[Asp^{10}]α-MSH	6	8.56×10^{-5} ± 5.14×10^{-5} n=3	9.98×10^{-8} ± 1×10^{-7} n=3
[Phe^{12}]α-MSH	7	6.19×10^{-6} ± 1.25×10^{-6} n=3	9.35×10^{-9} ± 3.73×10^{-9} n=3
[Asp^{10},Phe^{12}]α-MSH	8	7.59×10^{-5} ± 5.53×10^{-5} n=3	2.09×10^{-7} ± 3.08×10^{-7} n=3
γ_1-MSH	9	2.69×10^{-5} ± 1.02×10^{-5} n=4	4.35×10^{-8} ± 1.92×10^{-8} n=3
[Nle^3]γ_1-MSH	10	1.69×10^{-5} ± 1.99×10^{-6} n=3	4.42×10^{-7} ± 2.54×10^{-7} n=4
N$^{\alpha}$-Lys-[Nle^3]γ_1-MSH	11	3.30×10^{-5} ± 7.21×10^{-6} n=3	1.12×10^{-6} ± 4.11×10^{-7} n=3
[Nle^3,Pro^{11}]γ_1-MSH	12	1.95×10^{-5} ± 1.24×10^{-5} n=3	4.43×10^{-8} ± 3.8×10^{-8} n=3
[Nle^3,Ser^{11}]γ_1-MSH	13	4.71×10^{-5} ± 2.47×10^{-5} n=3	1.55×10^{-7} ± 8.96×10^{-8} n=3
[Nle^3,Leu^{11}]γ_1-MSH	14	3.41×10^{-5} ± 1.14×10^{-5} n=3	6.28×10^{-7} ± 4.26×10^{-7} n=3

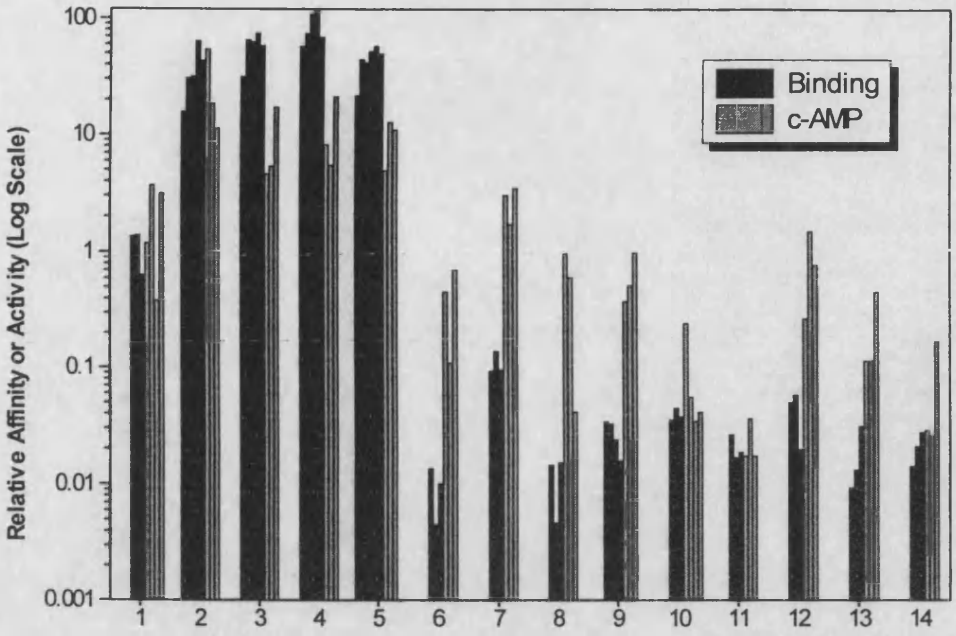


Figure.3.7B : Relative affinity or activity of α-MSH and γ_1-MSH analogues to α-MSH at MC3-R (log scale). Individual bars represent single experiments. Black = Affinity, Grey = Activity.

	No	Binding(Kd)	c-AMP(EC_{50})
α-MSH	1	1.0	1.
[Nle^4,D-Phe^7]α-MSH	2	30.5	19
[Nle^4,D-Phe^7,Phe^{12}]α–MSH	3	54.0	6.6
[Nle^4,D-Phe^7,Ser^{12}]α-MSH	4	80.3	8.6
[Nle^4,D-Phe^7,Leu^{12}]α-MSH	5	40.7	8.17
[Asp^{10}]α-MSH	6	0.008	0.24
[Phe^{12}]α-MSH	7	0.106	2.5
[Asp^{10},Phe^{12}]α-MSH	8	0.009	0.11
γ_1-MSH	9	0.025	0.54
[Nle^3]γ_1-MSH	10	0.039	0.05
N$^{\alpha}$-Lys-[Nle^3]γ_1-MSH	11	0.02	0.02
[Nle^3,Pro^{11}]γ_1-MSH	12	0.034	0.53
[Nle^3,Ser^{11}]γ_1-MSH	13	0.014	0.152
[Nle^3,Leu^{11}]γ_1-MSH	14	0.019	0.038

Table.3.3B. Relative affinity constants and EC_{50} values for α-MSH analogues at MC3-R.

3.2.2. Analogues with Multiple Alanine Substitutions

3.2.2.1. MC1 Receptor

Analogues with multiple alanine substitutions within the C-terminus (compounds **3-6**) (Table.3.4A and Table 3.4B.) showed that both affinity and activity of the peptides were decreased significantly from 5% to 50% for affinity and from 0.7% to 9% for activity when compared to α-MSH. In particular, substitutions in position 11 and 12 reduced the affinity to 5% and activity to 0.7% for the MC1-R compared to α-MSH. Compound **6** had less lost affinity while reducing the activity significantly to 0.7% of the α-MSH value. Among the compounds **3-6**, [Ala^{11},Ala^{13}]α-MSH showed the least reduced affinity and activity (Fig. 3.5).

	No	**Binding(K_d)**	**c-AMP (EC_{50})**
α-MSH	**1**	1.68×10^{-8} $\pm 3.88 \times 10^{-9}$ n=5	2.74×10^{-9} $\pm 9.56 \times 10^{-10}$ n=6
[Nle^4,D-Phe^7]α-MSH	**2**	3.27×10^{-9} $\pm 1.78 \times 10^{-9}$ n=5	1.67×10^{-10} $\pm 1.30 \times 10^{-10}$ n=6
[Ala^{11},Ala^{12}]α-MSH	**3**	3.53×10^{-7} $\pm 8.02 \times 10^{-8}$ n=3	3.79×10^{-7} $\pm 2.68 \times 10^{-7}$ n=3
[Ala^{11},Ala^{13}]α-MSH	**4**	3.44×10^{-8} $\pm 1.62 \times 10^{-8}$ n=3	2.99×10^{-8} $\pm 1.51 \times 10^{-8}$ n=3
[Ala^{12},Ala^{13}]α-MSH	**5**	4.33×10^{-8} $\pm 2.22 \times 10^{-8}$ n=3	7.26×10^{-8} $\pm 1.24 \times 10^{-8}$ n=3
[Ala^{11},Ala^{12},Ala^{13}]α-MSH	**6**	1.24×10^{-7} $\pm 3.46 \times 10^{-8}$ n=3	3.73×10^{-7} $\pm 1.91 \times 10^{-7}$ n=3

Table.3.4A : Molar dissociation constants, EC_{50} values, and their standard deviation for Alanine analogues at B16 Melanoma Cell (Statistics data, appendix 1.5 & 1.6).

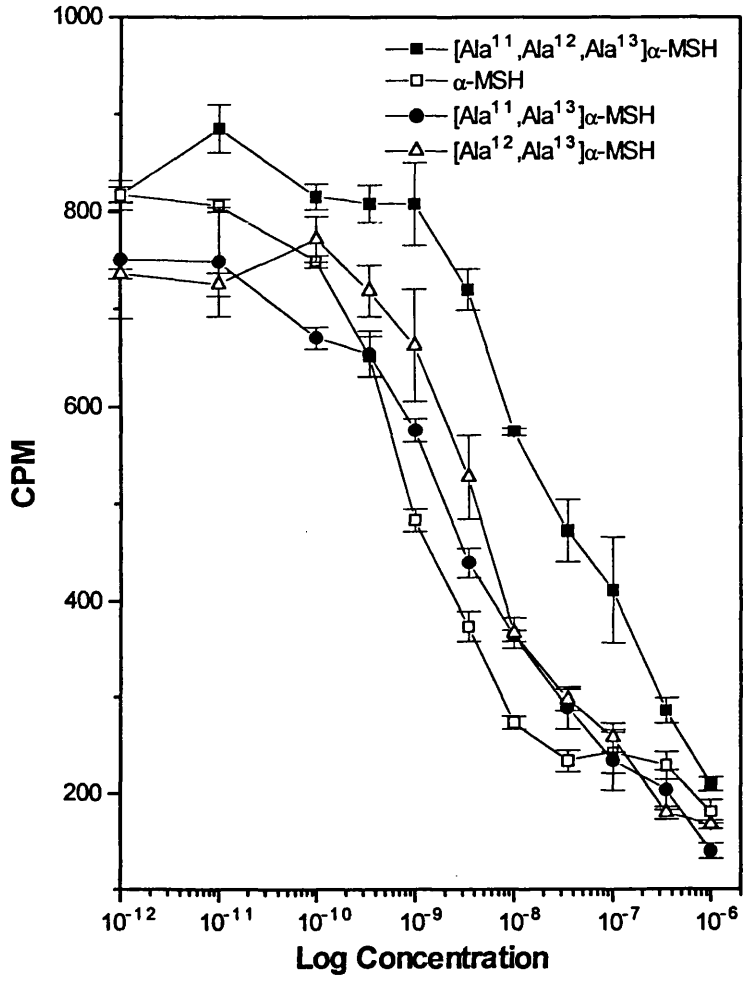


Figure.3.5A : Displacement of [^{125}I-Tyr^2, Nle^4, D-Phe^7]α-MSH from the MC1-R by alanine analogues and α-MSH.

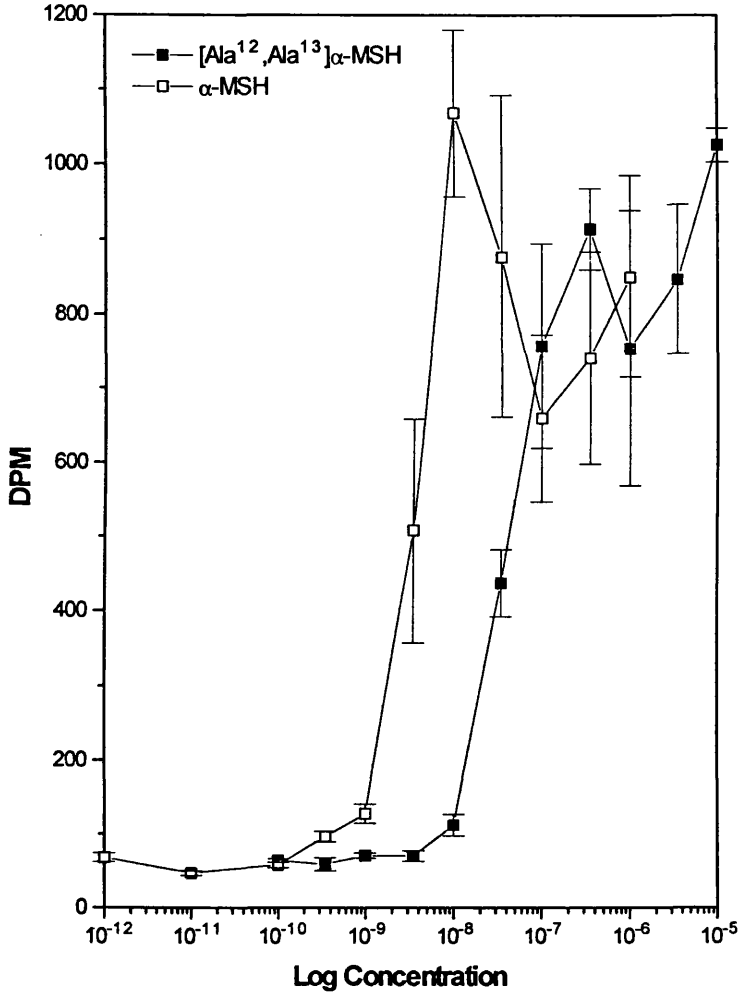


Figure.3.5B : Stimulation of cAMP production at MC1-R by alanine analogues and α-MSH.

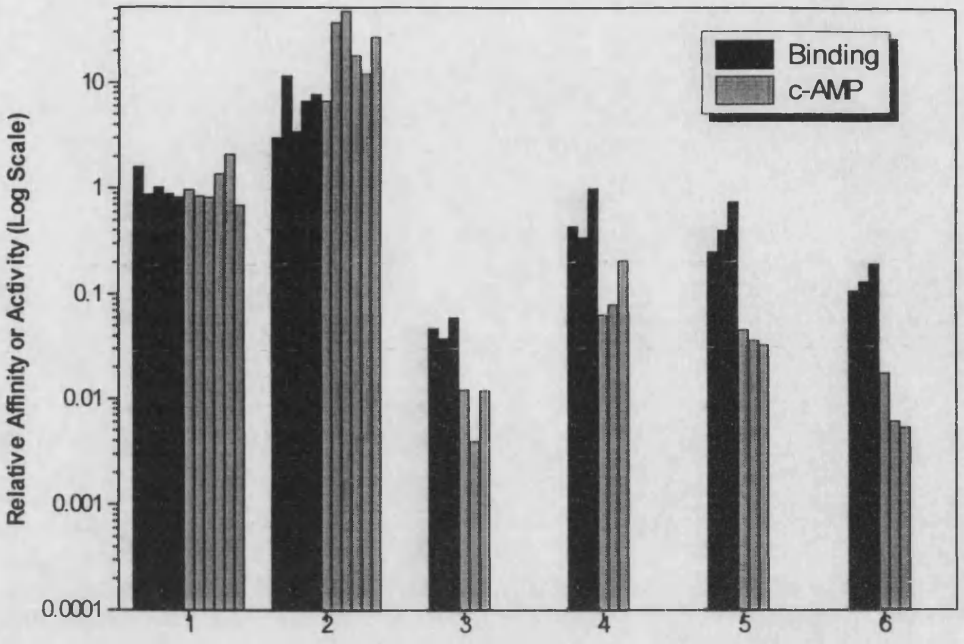


Figure.3.8A : Relative affinity or activity alanine analogues of α-MSH at MC1-R (log scale). Individual bars represent single experiments. Black = Affinity, Grey = Activity.

B16	No.	Binding (Kd)	c-AMP (EC_{50})
α-MSH	**1**	1.0	1.0
[Nle^4,D-Phe^7] α-MSH	**2**	5.14	16.4
[Ala^{11},Ala^{12}]α-MSH	**3**	0.048	0.007
[Ala^{11},Ala^{13}]α-MSH	**4**	0.49	0.09
[Ala^{12},Ala^{13}]α-MSH	**5**	0.39	0.04
[Ala^{11},Ala^{12},Ala^{13}]α-MSH	**6**	0.14	0.007

Table 3.4B : Relative affinity constants and EC_{50} values for alanine analogous at B16 Melanoma Cell.

3.2.2.2. MC3 Receptor

With multiple alanine substitutions (compounds **3-6**) (Table.3.5A,and Talbe.3.5B), there was to a significant drop in the binding to the receptor with relative affinities of 4-10% except [Ala^{12},Ala^{13}]α-MSH, which had similar properties to α-MSH. However, the biological activity of peptides with multiple alanine substitution at MC3-R was not significantly different from α-MSH. [Ala^{11}, Ala^{12}]α-MSH demonstrated that the lowest affinity to 4% and activity to 74% among compounds **3-6**. [Ala^{11}, Ala^{12}, Ala^{13}]α-MSH showed the lower affinity to 5% but the similar activity to α-MSH. [Ala^{11},Ala^{13}]α-MSH had affinity reduced to 10% but the activity was similar to α-MSH. In general, with multiple alanine substitutions, decreased binding affinity could be observed but activity at MC3-R was maintained (Fig 3.6).

MC3-293	**No**	**Binding(Kd)**	**cAMP(EC_{50})**
α-MSH	**1**	7.56×10^{-7} $\pm 7.87 \times 10^{-8}$ n=3	2.36×10^{-8} $\pm 2.55 \times 10^{-8}$ n=4
[Nle^4,D-Phe^7]α-MSH	**2**	3.27×10^{-9} $\pm 1.78 \times 10^{-9}$ n=5	1.67×10^{-10} $\pm 1.30 \times 10^{-10}$ n=6
[Ala^{11},Ala^{12}]α-MSH	**3**	1.95×10^{-5} $\pm 3.26 \times 10^{-6}$ n=5	3.21×10^{-8} $\pm 7.65 \times 10^{-9}$ n=4
[Ala^{11},Ala^{13}]α-MSH	**4**	7.27×10^{-6} $\pm 2.59 \times 10^{-6}$ n=5	2.82×10^{-8} $\pm 1.1 \times 10^{-8}$ n=5
[Ala^{12},Ala^{13}]α-MSH	**5**	1.06×10^{-6} $\pm 2.01 \times 10^{-7}$ n=3	5.62×10^{-9} $\pm 3.52 \times 10^{-9}$ n=3
[Ala^{11},Ala^{12},Ala^{13}]α-MSH	**6**	1.42×10^{-5} $\pm 4.85 \times 10^{-6}$ n=3	1.57×10^{-8} $\pm 3.99 \times 10^{-9}$ n=3

Table.3.5A. Molar dissociation constants, EC_{50} values, and their standard deviation for Alanine analogues at MC3-R cell (Statistics data, appendix 1.7 & 1.8).

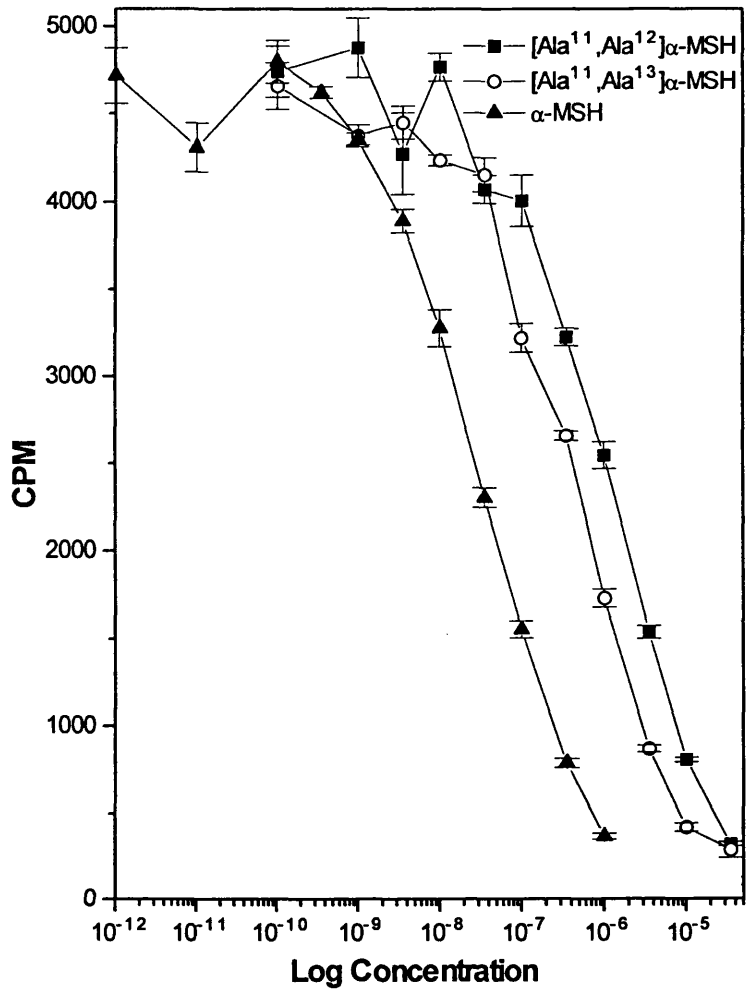


Figure.3.6A : Displacement of [^{125}I-Tyr^2, Nle^4, D-Phe^7]α-MSH from the MC3-R by alanine analogues and α-MSH.

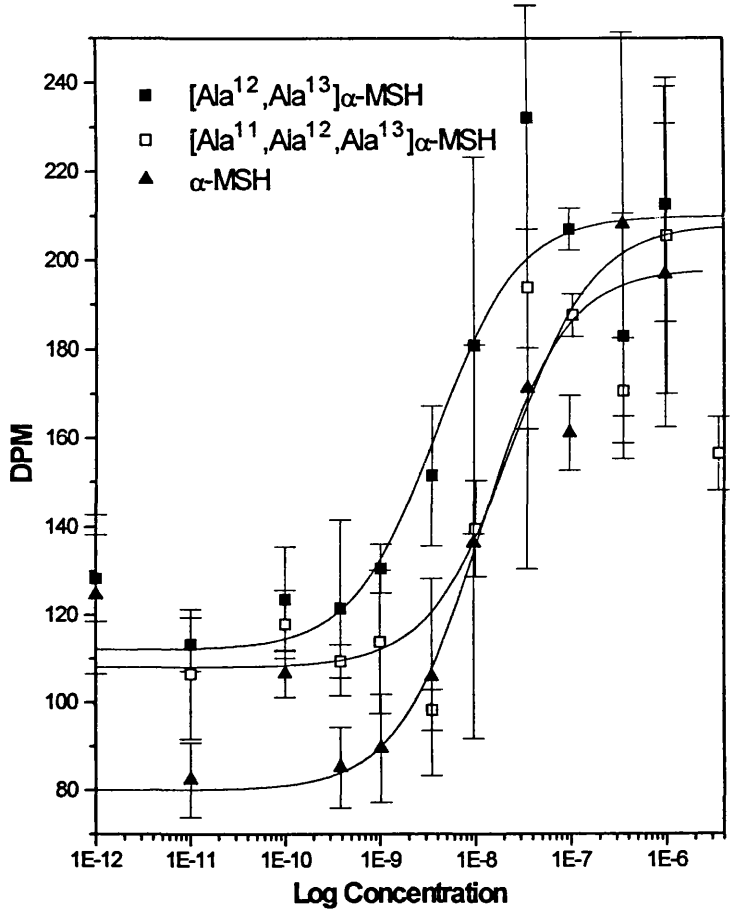


Figure.3.6B : Stimulation of cAMP production at MC3-R by alanine analogues and α-MSH.

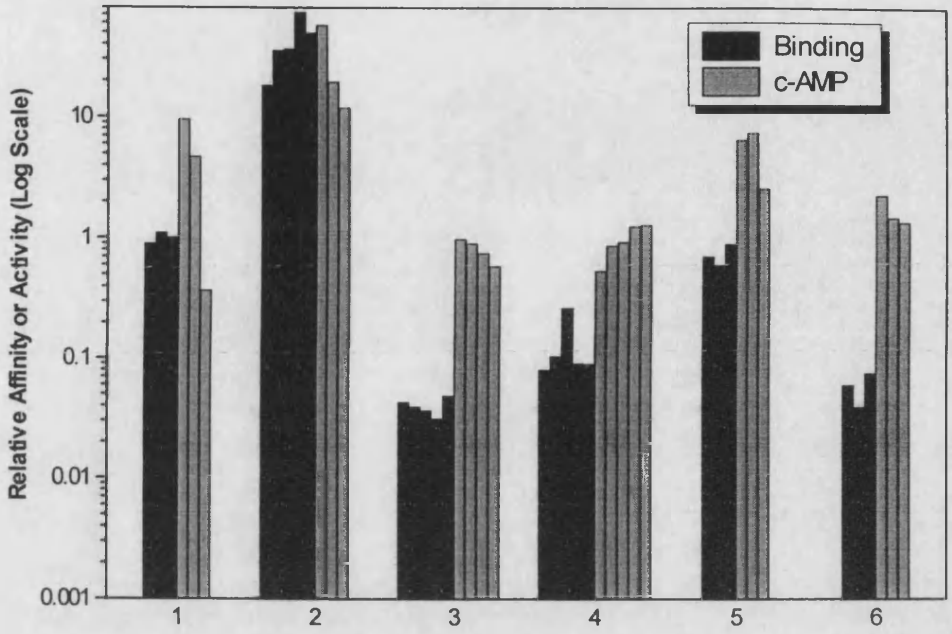


Figure.3.8B : Relative affinity or activity alanine analogues of α-MSH at MC3-R (log scale). Individual bars represent single experiments. Black = Affinity, Grey = Activity.

MC3-293	No.	Binding (K_d)	c-AMP(EC_{50})
α-MSH	**1**	1.0	1.0
[Nle^4,D-Phe^7] α-MSH	**2**	35	19
[Ala^{11},Ala^{12}]α-MSH	**3**	0.039	0.74
[Ala^{11},Ala^{13}]α-MSH	**4**	0.104	0.84
[Ala^{12},Ala^{13}]α-MSH	**5**	0.713	4.2
[Ala^{11},Ala^{12},Ala^{13}]α-MSH	**6**	0.053	1.5

Table 3.5B : Relative affinity constants and EC_{50} values for alanine analogous at MC3-R cells.

3.3. DISCUSSION

The "core" sequence of α-MSH, 6-9, has been identified as a basic sequence for melanotropic activity. Indeed, the tetrapeptide alone still retains some activity[39]. Any substitution within the 6-9 region, except with D-Phe^7 and/or D-Trp^9, leads to a loss of activity and affinity. Therefore it could not provide selectivity for one subclass of MSH receptors..

The N-terminus, Ser-Tyr-Ser, does not appear to have activity on its own. Therefore it is not likely to confer any selectivity.

The C-terminus of α-MSH, Lys-Pro-Val, provides distinct activity alone although it is not competitive as core sequence. Thus, the C-terminus could be a place to confer some selectivity of agonist or antagonist activity for α-MSH and γ-MSH.

In a previous study, Sahm *et al.*[135] substituted every position of α-MSH with alanine, and it appeared that position 12 conferred some degree of selectivity between MC1-R and MC3-R. As the MC1-R has strong binding with α-MSH and γ_1-MSH has by comparison better binding affinity with the MC3-R, we included γ_1-MSH in the study.

Substitutions made were in position 12 by Phe, Ser, and Leu on [Nle^4,D-Phe^7]α-MSH and position 11 with Pro, Ser, and Leu on [Nle^3]γ_1-MSH. Substitution of Pro by Phe in α-MSH and Phe by Pro in γ_1-MSH were made because they correspond to each other in the different peptides, and it was proposed that they are important for selectivity. The reason for substitution with Ser^{12} was that Ser has the closest hydrophobicity to proline but does not have a cyclic structure, and the reason for substitution with Leu is that Leu has stronger hydrophobicity than Pro, but likewise no cyclic structure.

When analysing the data it was observed that modification of the melanocortin peptide often led to analogues where either affinity or activity, but not both, were significantly changed. This is of interest in establishing the requirements for

stimulation of each receptor. It was therefore useful to find a comparative measure for the peptides' efficacy at each receptor by calculating the ratio between their activity and their affinity for each ligand (Fig 3.9A + 3.9B and 3.11A + 3.11B). Another purpose of the study was to establish the requirements for ligand binding selectivity which implies a relative difference in affinity of ligands for different subclasses of receptor. Therefore, to enable the comparative analysis between the receptors, selectivity of the analogues was determined by calculating a ratio between their respective affinities for each receptor (Fig. 3.10 and 3.12). Both of these ratios are expressed relative to α-MSH to facilitate comparison.

At the murine MC1-R, all analogues of [Nle^4,D-Phe^7]α-MSH bound with equal affinity and exhibited the same biological activities irrespective of their substitution at position 12; this effect was not be observed for α-MSH itself. It has been postulated that analogues of [Nle^4,D-Phe^7]α-MSH might interact with the MC1-R in a different way to α-MSH[145] and the results presented in this study support this hypothesis. However, it has to be noted that [Nle^4,D-Phe^7,Phe^{12}]α-MSH was the only one of the series that showed decreased efficacy compared to both α-MSH and the other [Nle^4,D-Phe^7]α-MSH analogues (Fig3.9A).

α-MSH, when substituted with phenylalanine in position 12, showed a significant reduction of both affinity and activity; it was therefore concluded that position 12 might play an important role in the interaction of α-MSH with murine MC1-R. This is further supported by reports that substitution of proline with alanine[135], D-proline, norvaline and glycine[39] all significantly decreased affinity and/ or activity of the peptide at the MC1-R. It has been suggested previously that position 10 of α-MSH, glycine, might function as a "spacer" between the core sequence and that the C-terminus of the molecule could be of importance in the interaction with the receptor[141]. In the studies, glycine could be replaced cysteine[185] and lysine [146,150] ,both introduced to enable cyclisation, and alanine[135], without affecting the

peptides' properties; however, introduction of the acidic amino acid aspartate significantly reduced both receptor binding and biological activity. A peptide containing both substitutions, synthesised in analogy to γ-MSH, [Asp^{10},Phe^{12}]α-MSH (**8**) (Table 3.1), was virtually inactive with both its affinity and activity being lower than that of γ-MSH. All three peptides showed a reduced efficacy on the MC1-R compared to α-MSH.

Peptides with multiple alanine substitutions within the C-terminus all were less active than α-MSH; in the case of one, [Ala^{11},Ala^{13}]α-MSH (**4**) (Table.3.3), the biological activity was reduced by the replacement, but not its affinity for the receptor. Generally, the biological activity was more affected than the receptor binding, leading to peptides with decreased efficacy (Fig.3.11A). Substitution at either position 11 and 12 (Table 3.4A), or 11,12, and 13 had the greatest effect; in contrast, very little change was observed following substitutions at position 11 or 13. Therefore it was concluded that the residue with the most influence on receptor binding and biological activity is proline 12, followed by lysine 11. This is in agreement with previous studies using single substitutions with alanine in this region; in this study [Ala^{12}]α-MSH bound with significantly lower affinity than α-MSH, [Ala^{11}]α-MSH was nearly as active as the native peptide and no difference could be found between [Ala^{13}]α-MSH and α-MSH[135]. The reduced efficacy caused by multiple amino acid replacements in the C-terminus might also support the hypothesis that this region may contain a second message sequence. This is further supported by the significantly lower affinity and activity of α-MSH$_{1\text{-}10}$ reported in previous studies[39,134].

All γ-MSH peptides tested showed very low affinity to and activity at the MC1-R. This was expected from the literature[53,186]. The synthetic analogues, [Nle^3]γ$_1$-MSH and N$^{\alpha}$-Lys-[Nle^3]γ$_1$-MSH bound with even lower affinity than γ$_1$-MSH, and for [Nle^3]γ$_1$-MSH, it was not possible to determine accurately the biological activity of

this peptide. Of all γ-MSH peptides, only [Nle^3,Pro^{11}] γ_1-MSH was more potent than γ_1-MSH and its activity was comparable to that of [Asp^{10}]α-MSH, which it resembles structurally. It was also the only γ_1-MSH peptide tested that showed an increased selectivity for the MC1-R (Fig.3.10A). All C-terminally modified γ_1-MSH peptides showed an increased efficacy at the MC1-R compared to γ-MSH; but in all cases it was lower than that of α-MSH itself (Fig.3.9A).

The data obtained for the MC1-R support the hypothesis that proline 12 (numbering with respect to α-MSH) is an important residue for recognition at the mouse MC1-R. Replacement of this position with any residue will influence the properties of α-MSH and γ-MSH analogues as long as they contain L-phenylalanine in position 7. However, it appears that introduction of phenylalanine in position 12 will affect the peptide in a particularly adverse manner.

At the MC3-R, as at the MC1-R, all analogues of [Nle^4,D-Phe^7]α-MSH showed higher affinity and increased biological activity (Table 3.2 and 3.3B). C-terminal substitutions exerted no visible effects on the potency at the MC3-R. [Nle^4,D-Phe^7]α-MSH and all its analogues showed reduced efficacy compared to α-MSH (Fig.3.9B), however, their selectivity for the MC3-R was slightly increased (Fig.3.10B).

Substitution of position 12 with phenylalanine (compound 7) (Table 3.2) decreased the affinity of α-MSH, but left its activity virtually unchanged. The replacement of glycine 10 with aspartate had a more pronounced effect on both affinity and activity. The peptide with both substitutions, [Asp^{10},Phe^{12}]α-MSH, compound 8, behaved in a similar way to the peptide with only position 10 changed. It has to be noted that the latter two peptides showed an increased selectivity for the rat MC3-R in comparison with α-MSH (Fig 3.10B).

Multiple substitutions with alanine in the C-terminal region (compounds **3-6**) (Table 3.5.B) failed to result in peptides with significantly different activity from α-MSH, although for most of them a slight reduction in binding affinity could be observed. In a previous study, single amino acid replacements with alanine failed to yield peptides with significantly different binding activities from α-MSH[135,181]. However, in another study, it was shown that $ACTH_{1-10}$ showed an approximately 50 times lower biological activity than $ACTH_{1-13}$ (which is equivalent to α-MSH)[184]; therefore the C-terminal end of the peptide must have a role in interacting with the MC3-R. Also, it was observed that all C-terminally modified L-Phe^7 analogues of α-MSH showed a greater efficacy than α-MSH at the MC3-R (Fig. 3.9B). As this could not be seen with analogues of [Nle^4,D-Phe^7]α-MSH, it opens the question whether [Nle^4,D-Phe^7]α-MSH and its analogues might interact in a different manner from α-MSH with this MC3-R as has been postulated for the MC1-R[145].

γ-MSH peptides bound to the rat MC3-R with reduced affinity compared to α-MSH, but their biological activity was in the same range as that of α-MSH (Table 3.3). From that if follows that γ-MSH peptides have an increased efficacy at the MC3-R versus α-MSH; of all γ-MSH peptides tested, [Nle^3,Leu^{11}]γ_1-MSH (compound **14**) was the analogue with the lowest potency and efficacy. Substitution of phenylalanine 11 with proline (in analogy to α-MSH) did not affect the activity of the peptide; this was in agreement with a study in which Miwa *et al*[184]. concluded that the position 11 of γ-MSH might not significantly contribute to the biological activity at the MC3-R, however in this study we observed that all γ-MSH except [Nle^3,Pro^{11}]γ_1-MSH showed increased selectivity for MC3-R. [Nle^3,Pro^{11}]γ_1-MSH was not more selective for the MC3-R than α-MSH. This together with the reduced activity observed for $ACTH_{1-10}$ at the MC3-R[184] might

well indicate a role for the C-terminal end of melanocortin peptides in the interaction with MC3-R. Also, the observation of increased efficacy and selectivity of γ-MSH peptides at the MC3-R supports the suggestion originally made by Roselli-Rehfuss *et al.*[62] that γ-MSH might be the natural ligand for the MC3-R.

The results reported here support the suggestion of a different manner of interaction for [Nle^4,D-Phe^7]α-MSH and its analogues with melanocortin receptors as has already been postulated for the human MC1-R[145]. These authors present further evidence for the crucial role of proline 12 in the interaction of α-MSH with the MC1-R. However, these results are in contradiction to the hypothesis that the C-terminus of melanocortin peptides might play a limited role in the recognition of and activity at the MC3-R[184]. The role of the C-terminal amino acids will need to be re-examined in relation to both α-MSH and γ-MSH. Overall, it can be concluded that the C-terminal amino acids play a pivotal role in differentiating between MC1-R and MC3-R subtypes and it is likely that this will also be the case at the MC4-R and MC5-R subtypes. In fact, this has already been suggested for the MC4 -R[184].

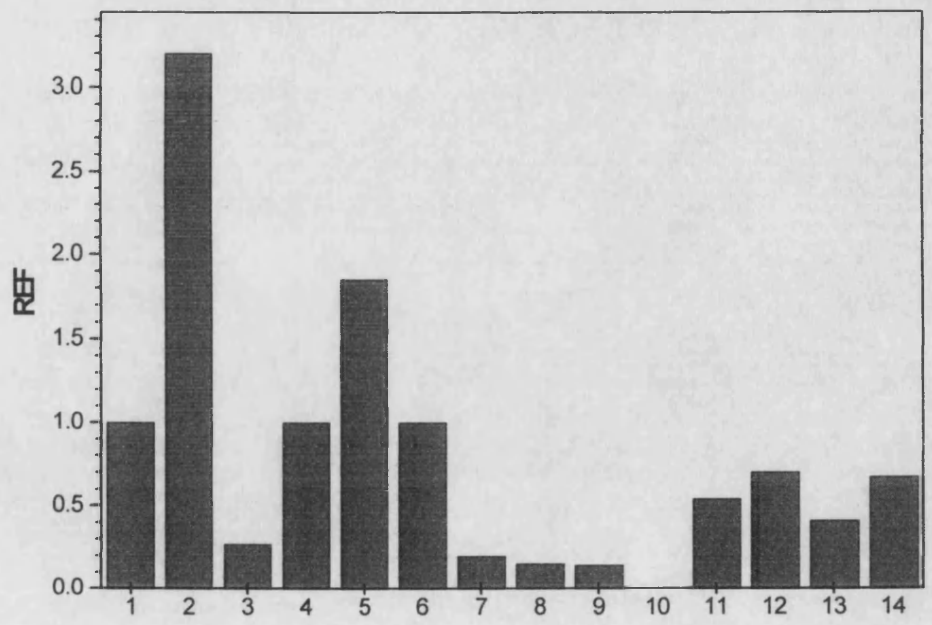


Figure.3.9A : Relative efficacy function (REF) at α-MSH analogues for MC1-R referring to Table3.1.

$$REF = \frac{Kd/EC_{50\ test}}{Kd/EC_{50\ \alpha\text{-MSH}}}$$

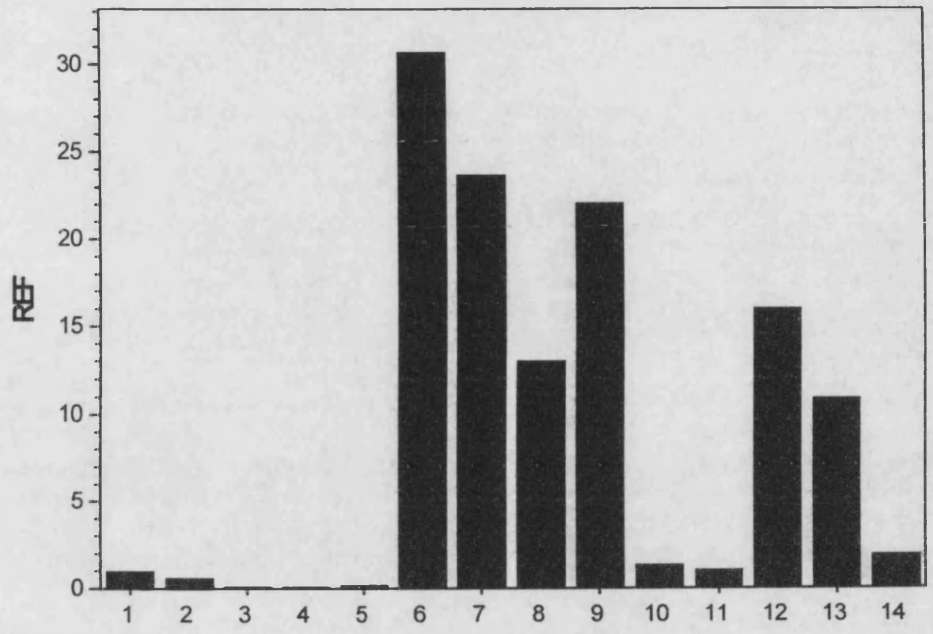


Figure.3.9B : Relative efficacy function (REF) at α-MSH analogues for MC3-R referring to Table3.2.

$$REF = \frac{Kd/EC_{50\ test}}{Kd/EC_{50\ \alpha\text{-MSH}}}$$

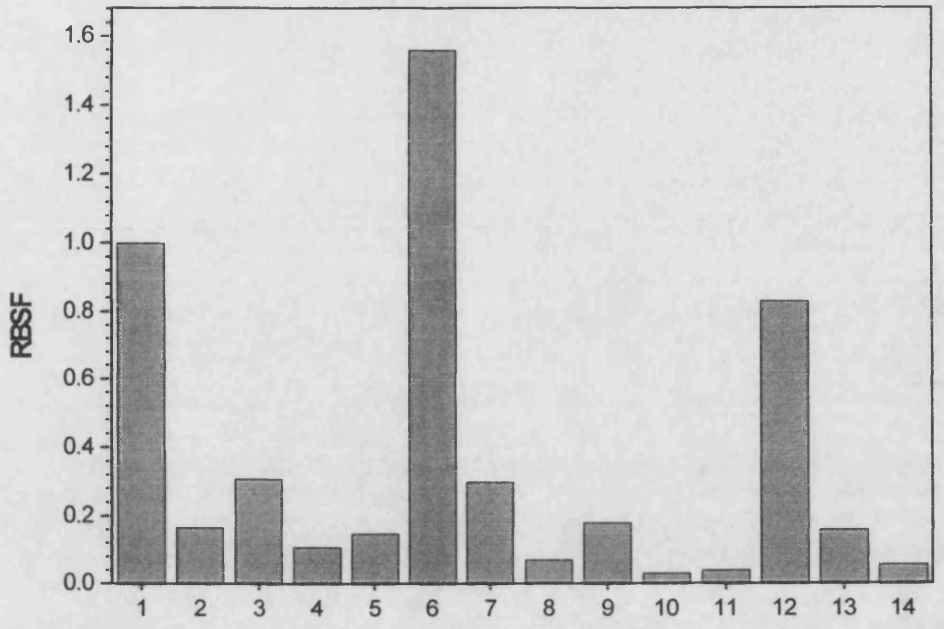


Figure.3.10A : Relative binding selectivity function (RBSF) at α-MSH analogues for MC1-R.

$$\text{RBSF} = \frac{Ka_{(test)}\,MC1/Ka_{(test)}MC3}{Ka_{(\alpha\text{-MSH})}\,MC1/Ka_{(\alpha\text{-MSH})}\,MC3}$$

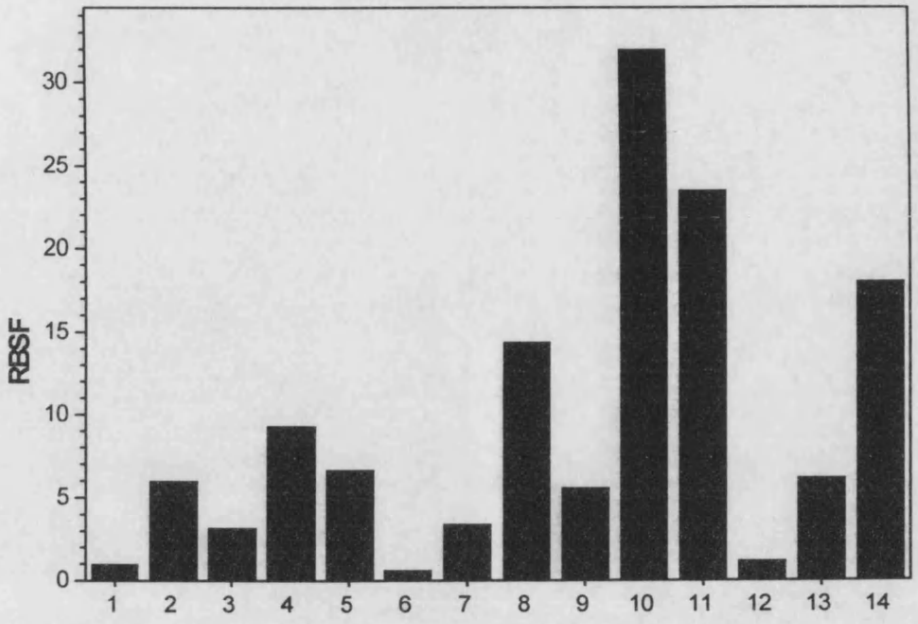


Figure.3.10B : Relative binding selectivity function (RBSF) at α-MSH analogues for MC3-R.

$$\text{RBSF} = \frac{Ka_{(test)}\,MC3/Ka_{(test)}MC1}{Ka_{(\alpha\text{-MSH})}\,MC3/Ka_{(\alpha\text{-MSH})}\,MC1}$$

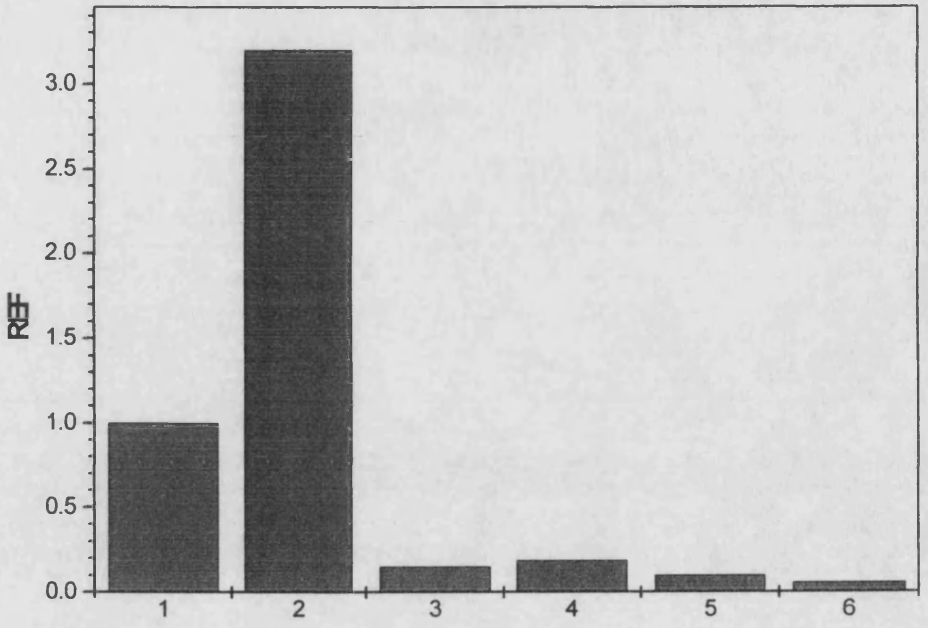


Figure.3.11A : Relative efficacy function (REF) at alanine analogues for MC1-R referring to Table 3.4A.

$$REF = \frac{Kd/EC_{50\ test}}{Kd/EC_{50\ \alpha\text{-MSH}}}$$

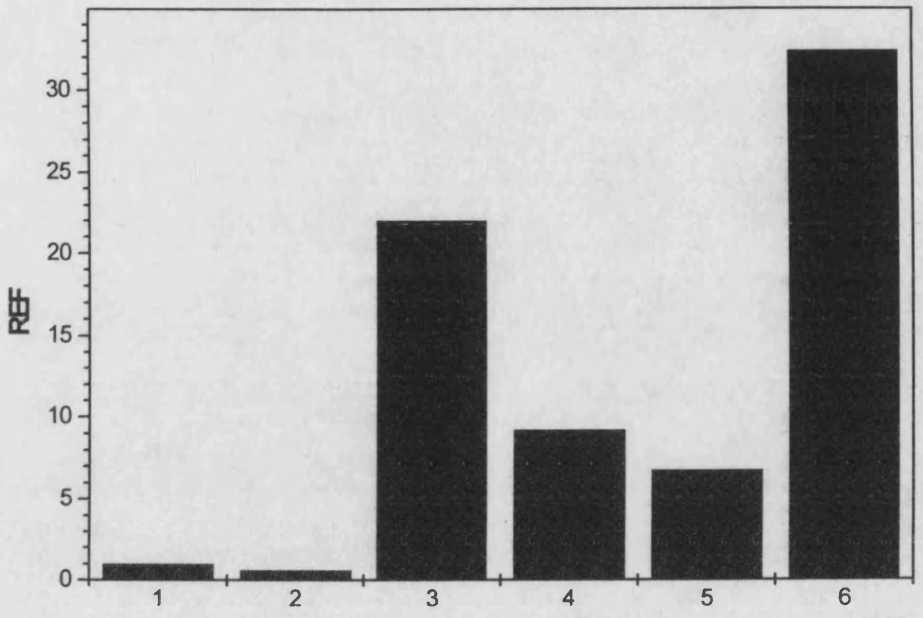


Figure.3.11B : Relative efficacy function (REF) at alanine analogues for MC3-R referring to Table 3.5A.

$$\text{REF} = \frac{\text{Kd/EC}_{50\ \text{test}}}{\text{Kd/EC}_{50\ \alpha\text{-MSH}}}$$

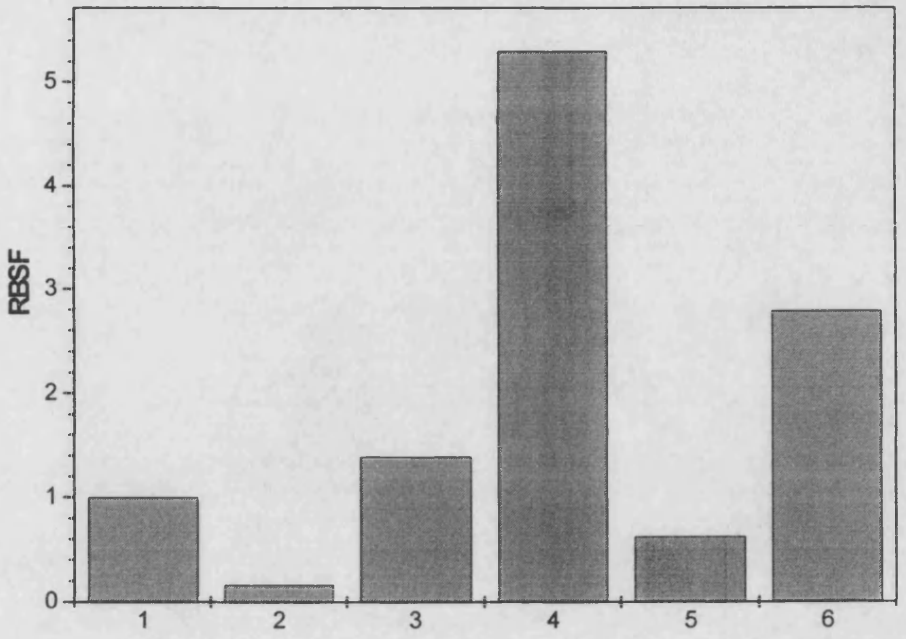


Figure.3.12A : Relative binding selectivity function (RBSF) at alanine analogues for MC1-R.

$$\text{RBSF} = \frac{Ka_{(test)}\,MC1/Ka_{(test)}MC3}{Ka_{(\alpha\text{-MSH})}\,MC1/Ka_{(\alpha\text{-MSH})}\,MC3}$$

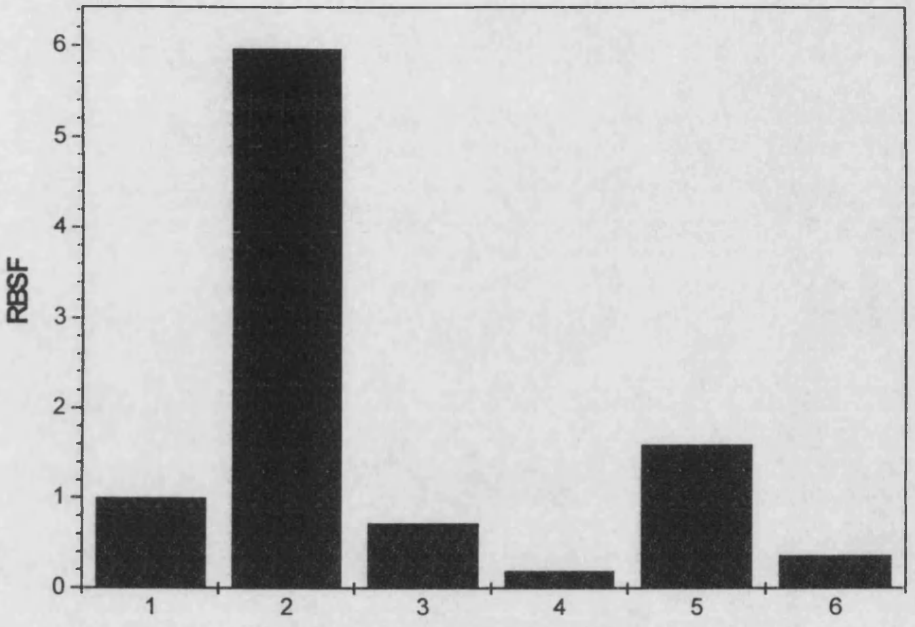


Figure.3.12B : Relative binding selectivity function (RBSF) at alanine analogues for MC3-R.

$$\text{RBSF} = \frac{Ka_{(test)}\,MC3/Ka_{(test)}MC1}{Ka_{(\alpha\text{-MSH})}\,MC3/Ka_{(\alpha\text{-MSH})}\,MC1}$$

Chapter 4: Activity of Melanocortins Modified at position 7 at the MC1, MC3, and MC4 Receptors

4.1. Introduction

The studies in this chapter focus on the importance of position 7 of α-MSH at the receptors MC1-R, MC3-R and MC4-R, and in particular, the effects of altering the sequence at position 7 and subsequent effects on ligand binding and cAMP production.

Hofmann *et al.*[193] first demonstrated that the entire 13 amino acid sequence of α-MSH is not required for melanotropic activity by synthesis of a bioactive peptide, Met-Glu-His-Phe-Arg-Trp-Gly. Furthermore, Yajima *et al.*[194] found that the His-D-Phe-Arg-Trp-Gly fragment showed a greater activity than an analogue containing only L-amino acids *in vitro* frog skin assay. Introduction of a second D-residue is only possible in position 9[133]. Replacement of D-His or D-Arg rendered the peptide inactive. Substituting more than two amino acids with their D-isomers as in D-His-D-Phe-D-Arg-Trp-Gly and His-D-Phe-D-Arg-D-Trp-Gly gave peptides without activity, but were weakly inhibitory when administered at the same time as the L-L-L-L- pentapeptide[131], while the all D-isomer showed even stronger inhibitory effects[194].

Later the "superpotent" agonist of α-MSH, [Nle^4,D-Phe^7]α-MSH, was discovered and shown to be 26 times as potent as α-MSH in the mouse melanoma cAMP assay[23]. A single injection of [Nle^4,D-Phe^7]α-MSH into frogs caused near maximum darkening of the skins for at least 6 weeks[26]. Injections of the natural hormone α-MSH or [Nle^4]α-MSH also caused darkening, but this effect lasted

only a few days[26]. [Nle^4,D-Phe^7]α-MSH may therefore induce tighter (apparently irreversible) binding to its receptor, leading to a sustained, biological activation of adenylate cyclase and cAMP production[26,145].

Wilkes *et al.*[195] have investigated the [Phe^7] position in the Ac-[Nle^4]α-MSH$_{4-11}$-NH$_2$ analogue. In order to investigate the influence of this particular amino acid, replacement of [Phe^7] by tyrosine, p-nitrophenylanine, alanine or glycine as well as by their corresponding D-amino acids was carried out. It was concluded that the D-Isomers were more potent than the L-forms in both the frog and lizard skin assays except for the [D-Ala^7] analogue. However, none of them were as potent as Ac-[Nle^4]α-MSH$_{4-11}$-NH$_2$ or Ac-[Nle^4,D-Phe^7]α-MSH$_{4-11}$-NH$_2$. Later, Hruby *et al.*[196] replaced Phe^7 with phenylglycine (Pgl) and 1,2,3,4-tetrahydroisoquinoline (Tic) and their D-isomers, but none of them were more potent than Ac-[Nle^4]α-MSH$_{4-11}$-NH$_2$, or Ac-[Nle^4,D-Phe^7]α-MSH$_{4-11}$-NH$_2$, except Ac-[Nle^4,Pgl^7]α-MSH$_{4-11}$-NH$_2$ in the frog skin assay. Replacement by D-Pgl led to a major reduction in activity in both frog and lizard bioassays whereas introduction of Tic affected the results in lizard skin significantly more than those from the frog skin assay[196].

Introduction of D-Phe^7 into the α-MSH molecule has been shown to make the molecular resistant to chemical degradation under *in vitro* and *in vivo* conditions, explaining the prolongation of effect and higher potency observed with [D-Phe^7]α-MSH analogues to [L-Phe^7]α-MSH[26,138,146].

[Met] can be replaced with [Nle] at position 4 of α-MSH because it is pseudoisosteric to [Met^4], but does not contain sulphur which is oxidised easily with loss of potency. Activity of [Nle^4]α-MSH is similar to α-MSH about 6 x 10^{-10}M in the frog skin assay[39].

Ac-[Nle^4,D-Phe^7]α-MSH$_{4-11}$-NH$_2$ has similar properties to [Nle^4,D-Phe^7]α-MSH in that it has high affinity, and in the tyrosinase assay the compound was 100-fold more active than α-MSH[147]. [Nle^4,D-Phe^7]α-MSH is stable to enzymatic degradation and is resistant to oxidative conditions found during radioiodination. Therefore it is routinely used as a standard in biological assays and facilitates radioiodination for use in binding assays.

Recently, Hruby *et al.*[159] have reported that substitution in the 7 position of Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$ can produce potent and selective antagonists for melanocortin receptors. Therefore, a series of substitution at 7 have been attempted. [D-p-iodophenylalanine^7]α-MSH was a potent antagonist at MC1-R in the frog skin(*Rana pipiens*) assay but a potent agonist at the human MC1-R and mouse MC1-R. In addition, it was a potent antagonist at the human MC3-R and MC4-R. D-2'-naphthylalanine 7 was a potent antagonist of the MC4-R and a less potent antagonist of the MC3-R and a full agonist of the MC1-R and MC5-R. Hruby *et al.*[159] indicated that modifications of the phenyl ring of the [D-Phe^7] residue of a cyclic lactam derivative of α-MSH$_{4-10}$ that retain aromatic character can result in melanocortin receptor antagonists with high potency and specificity. The aim of this chapter is to find more potent and receptor selective analogues by substitution at position 7. Replacements were made by [D/L-Tyr^7]α-MSH and [D/L-Trp^7]α-MSH and the peptides were tested at mouse MC1-R, rat MC3-R and rat MC4-R through binding and c-AMP assays.

4.2. Results

The B16 murine melanoma cells naturally expressing MC1-R and human embryo kidney cells expressing MC3-R and MC4-R were used to evaluate both the binding affinity and their ability to generate cAMP in response to the melanotropin peptides α-MSH, [D/L-Tyr^7]α-MSH, and [D/L-Trp^7]α-MSH. Results from binding and cAMP assays as well as values relative to α-MSH are given in Tables 4.1, 4.2, and 4.3 for the MC1-R, MC3-R and MC4-R, respectively. Fig 4.1 and 4.2 show the peptides binding activity at MC1-R; Fig 4.3, 4.4 give those at MC3-R; and Fig 4.5 shows their affinities and activities at MC4-R. Fig4.6A, 4.6B and 4.6C present the relative affinity and activity at MC1-R, MC3-R, and MC-4R, for each peptide examined in this study.

4.2.1. MC1 Receptor

[Nle^4,D-Phe^7]α-MSH had 2-fold higher affinity and 16-fold higher activity than α-MSH. Replacement of Phe^7 by [L/D-Tyr^7] or [L/D-Trp^7] led to loss of binding to the receptors and of the ability to activate cAMP production significantly (Table 4.1). In general, D-substituted peptides were more potent and active than L counterparts in B16 melanoma cells (Fig 4.1 and 4.2). However, α-MSH still remained more potent than D-substituted peptides. The only peptide to show similar properties to α-MSH was [D-Tyr^7]α-MSH (Fig 4.1A). [D-Trp^7]α-MSH had similar affinity (Fig 4.1B), but not activity to α-MSH. Biological activity was

also reduced significantly for [L-Tyr^7]α-MSH, [L-Trp^7]α-MSH and [D-Trp^7]α-MSH to 0.05%, 0.13%, and 17% respectively, but not for [D-Tyr^7]α-MSH.

B16	No	Binding (K_d/M)	c-AMP (EC_{50})
α-MSH	1	6.59×10^{-9} $\pm 9.97 \times 10^{-10}$ n=5	2.74×10^{-9} $\pm 9.56 \times 10^{-10}$ n=6
[Nle^4,D-Phe^7] α-MSH	2	3.27×10^{-9} $\pm 1.78 \times 10^{-9}$ n=5	1.67×10^{-10} $\pm 1.3 \times 10^{-10}$ n=6
[L-Tyr^7] α-MSH	3	1.90×10^{-7} $\pm 6.62 \times 10^{-8}$ n=3	5.13×10^{-6} $\pm 3.1 \times 10^{-6}$ n=4
[L-Trp^7] α-MSH	4	5.68×10^{-6} $\pm 2.37 \times 10^{-6}$ n=3	2.1×10^{-6} $\pm 4.45\ 10^{-7}$ n=4
[D-Tyr^7] α-MSH	5	4.27×10^{-9} $\pm 1.19\ 10^{-9}$ n=5	4.91×10^{-9} $\pm 3.81 \times 10^{-9}$ n=3
[D-Trp^7] α-MSH	6	1.53×10^{-8} $\pm 6.57 \times 10^{-9}$ n=4	1.63×10^{-8} $\pm 5.79 \times 10^{-9}$ n=3

Table 4.1A: Molar dissociation constants, EC_{50} values, and their standard deviation for [D/L-Tyr^7] / [D/L-Trp^7]α-MSH in B16 melanoma cells (Statistics data, appendix 1.9 & 1.10).

B16	No	K_d/M	EC_{50}
α-MSH	1.	1.0	1.0
[Nle^4,D-Phe^7] α-MSH	2	2.015	16.4
[L-Tyr^7] α-MSH	3	0.035	0.0005
[L-Trp^7] α-MSH	4	0.001	0.0013
[D-Tyr^7] α-MSH	5	1.543	0.56
[D-Trp^7] α-MSH	6	0.43	0.17

Table 4.1B: Binding affinity and biological activity of [D/L-Tyr^7] / [D/L-Trp^7]α-MSH in B16 melanoma cells relative to α-MSH.

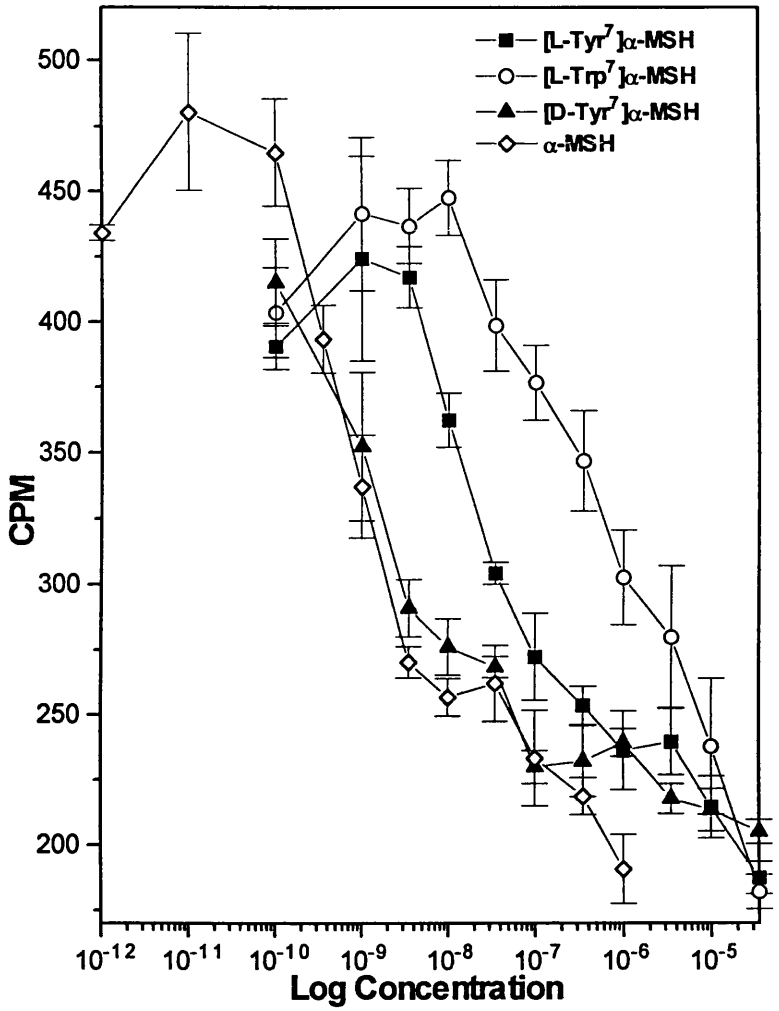


Figure 4.1A: Displacement of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH from the MC1-R.

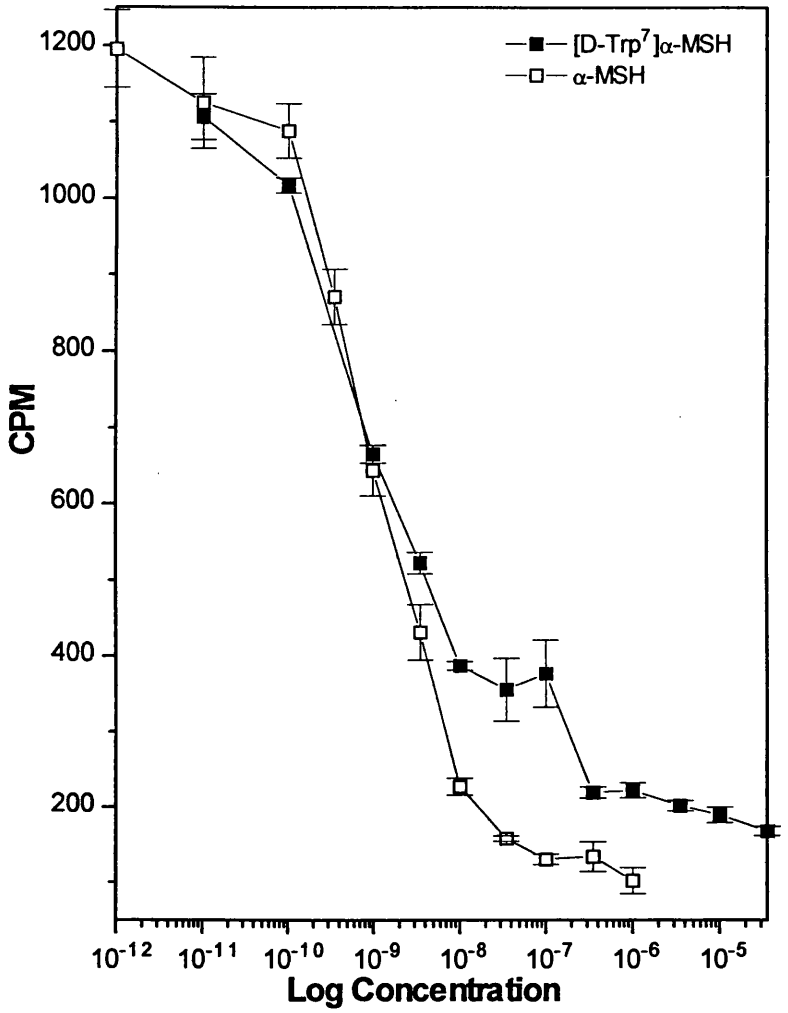


Figure 4.1B: Displacement of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH from the MC1-R.

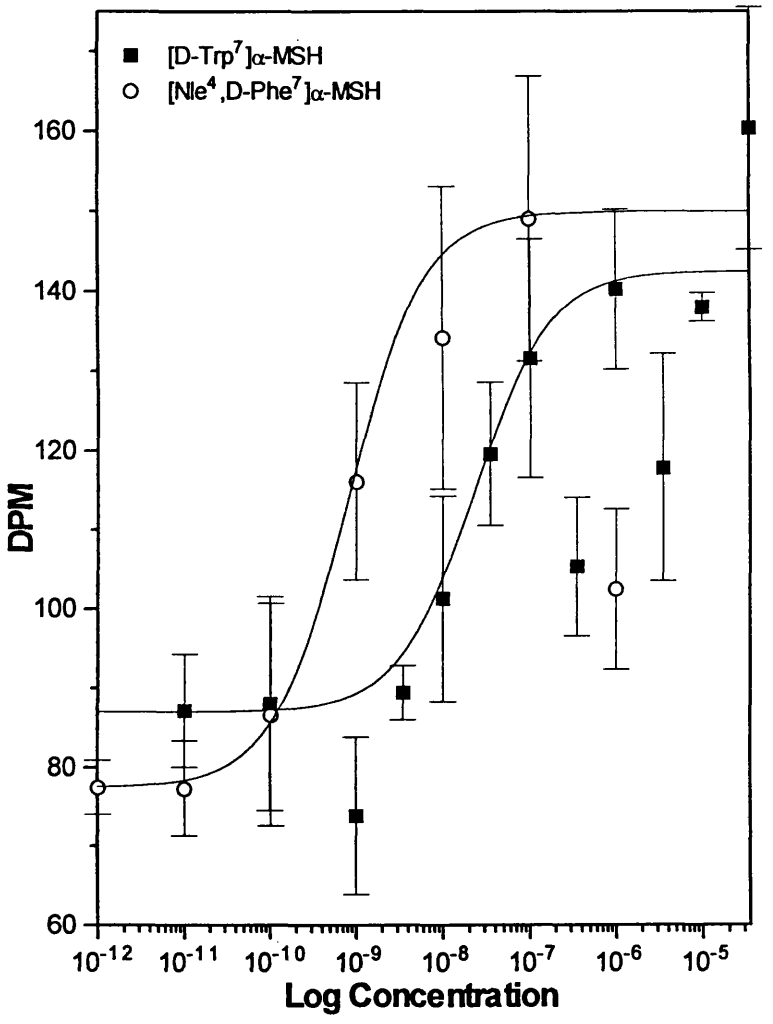


Figure 4.2A: Stimulation of cAMP production at MC1-R.

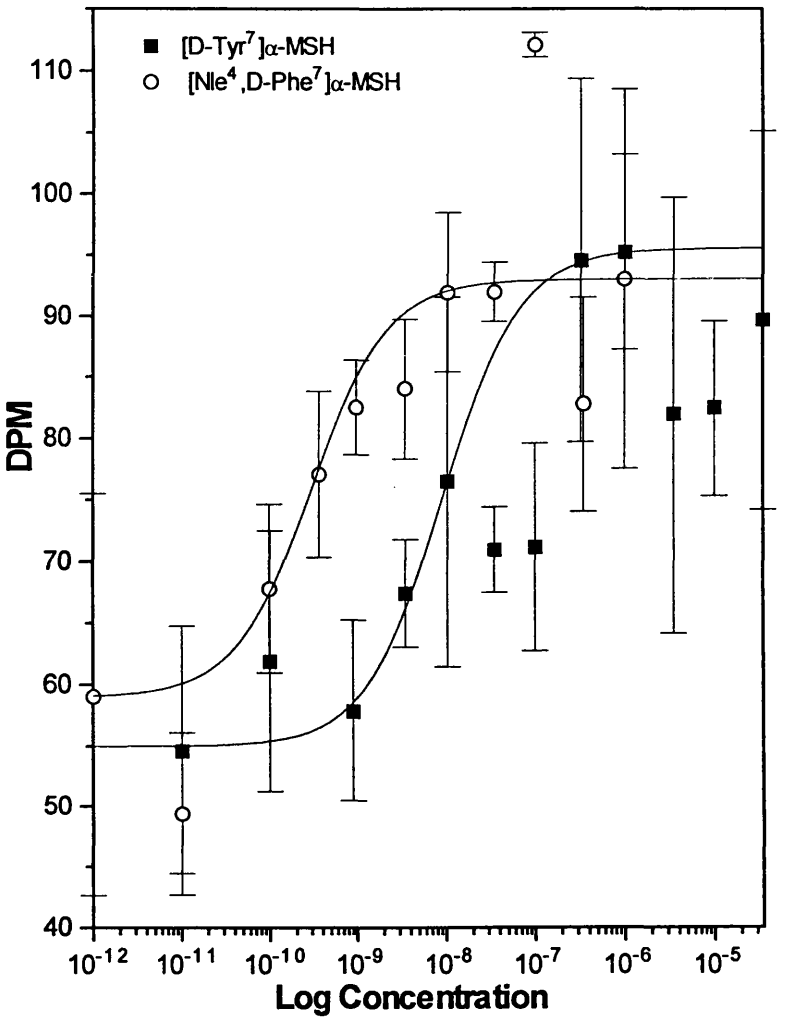


Figure 4.2B: Stimulation of cAMP production at MC1-R.

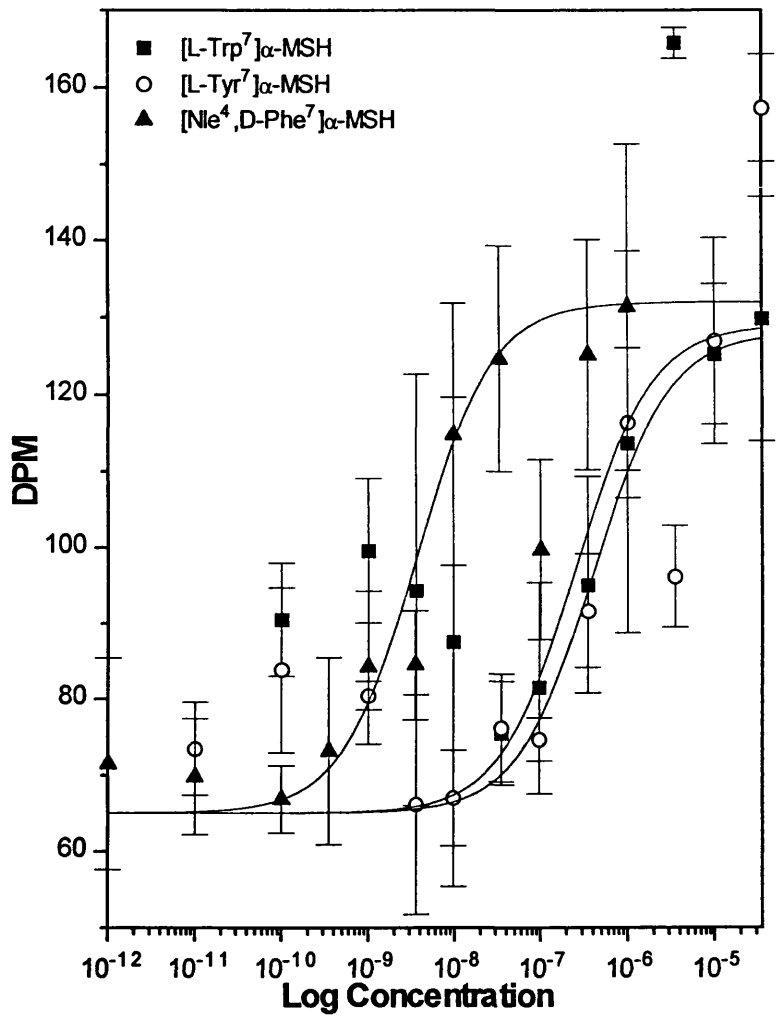


Figure 4.2C: Stimulation of cAMP production at MC1-R.

4.2.2. MC3 Receptor

Modifications of position 7 by [L/D-Tyr^7] and [L/D-Trp^7] gave very different results at the MC3-R compared to the MC1-R (Table 4.2A and 4.2B). [L-Tyr^7]α-MSH and [D-Tyr^7]α-MSH were able to stimulate cAMP production, whereas both [L-Trp^7]α-MSH and [D-Trp^7]α-MSH were inactive (Fig 4.3B + 4.3C). However, [D-Trp^7]α-MSH had the ability to antagonise the effect of α-MSH (Fig 4.4). [L-Tyr^7]α-MSH had affinity and activity significantly reduced to 1.4% and 1.8% compared to α-MSH, respectively. [L-Trp^7]α-MSH had very low affinity compared to that of α-MSH (3.1%) but was not able to exhibit biological activity at the MC3-R. [D-Tyr^7]α-MSH had a similar property to α-MSH (Fig 4.3A+4.3C). [D-Trp^7]α-MSH had a similar affinity to α-MSH but possessed the ability to antagonise α-MSH with an IC_{50} of 1.95 ± 0.628 x 10^{-7}M (Fig.4.4, Statistics data, appendix 1.13).

MC3-293	No	Binding (K_d/M)	c-AMP (EC_{50})
α-MSH	1	6.59 x 10^{-7}± 3.14 x10^{-7} n=3	2.36 x 10^{-8}± 2.55 10^{-8} n=4
[Nle^4,D-Phe^7]α-MSH	2	2.16 x 10^{-8}± 1.18x 10^{-8} n=5	1.25 x10^{-9}±8.20x10^{10} n=3
[L-Tyr^7] α-MSH	3	4.83 x 10^{-5}±5.13 x 10^{-6} n=4	1.28 x10^{-6}± 9.35x10^{-7} n=3
[L-Trp^7] α-MSH	4	2.09x 10^{-5}± 6.90 x 10^{-6} n=4	No activity
[D-Tyr^7] α-MSH	5	4.71 x 10^{-7}±1.55 x 10^{-7} n=4	2.66 x 10^{-8}± 8.90x10^{-9} n=3
[D-Trp^7] α-MSH	6	3.30 x 10^{-7}± 8.48 x 10^{-8} n=3	No activity

Table 4.2A: Molar dissociation constants, EC_{50} values, and their standard deviation for [D/L-Tyr^7]/ [D/L-Trp^7]α-MSH at MC3 receptor (Statistic data, appendix 1.11 & 1.12).

MC3-293	No	Kd	EC_{50}
α-MSH	1	1	1
[Nle^4,D-Phe^7] α-MSH	2	30.5	19
[L-Tyr^7] α-MSH	3	0.014	0.018
[L-Trp^7] α-MSH	4	0.031	No activity
[D-Tyr^7] α-MSH	5	1.4	0.9
[D-Trp^7] α-MSH	6	2	No activity

Table 4.2B: Binding affinity and biological activity of [D/L-Tyr^7]/ [D/L-Trp^7]α-MSH at MC3 receptor relative to α-MSH.

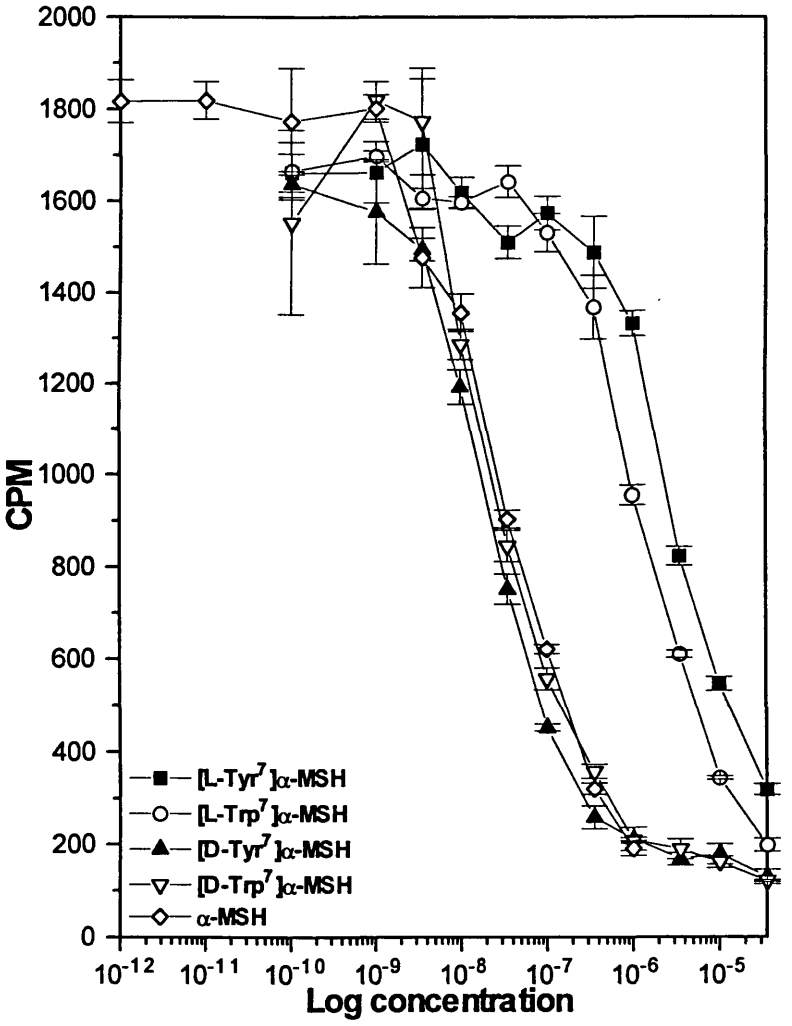


Figure 4.3A: Displacement of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH from the MC3-R.

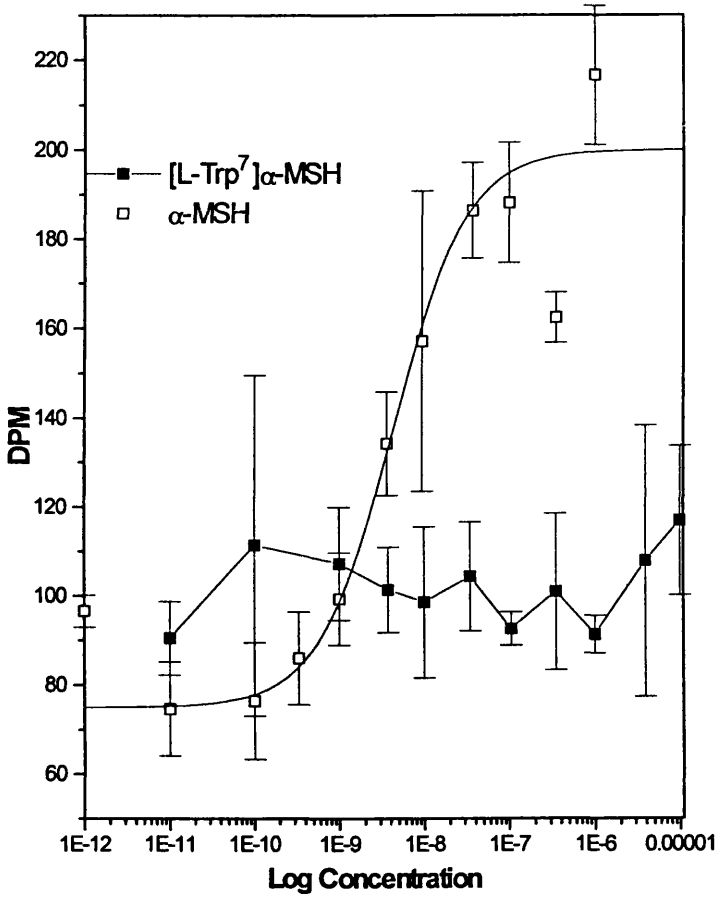


Figure 4.3B: Stimulation of cAMP production at MC3-R.

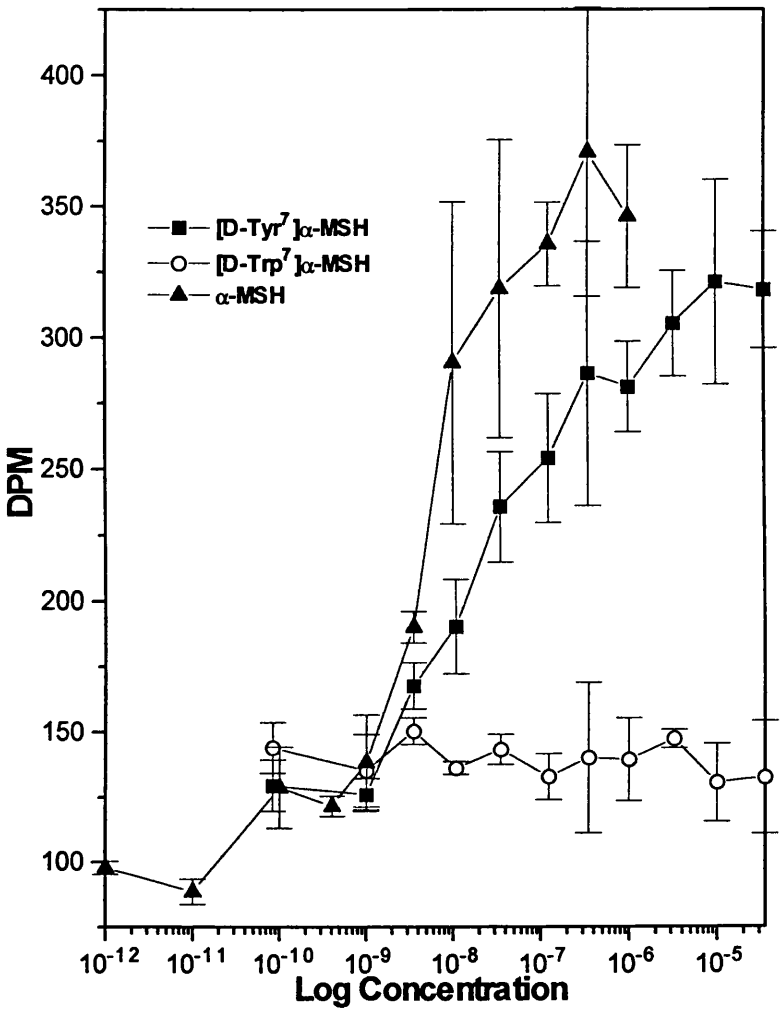


Figure 4.3C: Stimulation of cAMP production at MC3-R.

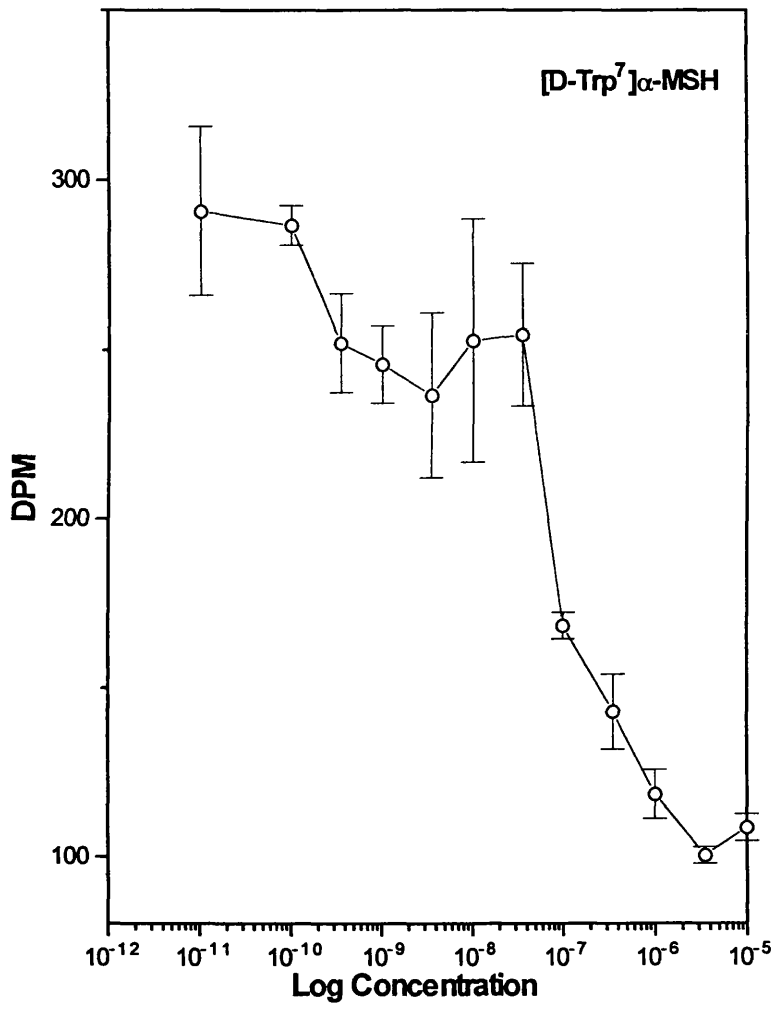


Figure 4.4: Stimulation of cAMP production at MC3-R. [D-Trp^7]α-MSH was an antagonist at MC3-R. Antagonist activity of [D-Trp^7] α-MSH was determined in the presence of 10^{-8} M α-MSH.

4.2.3. MC4 Receptor

Because of their interesting properties at the MC3-R, [L-Trp^7] and [D-Trp^7]α-MSH were also tested for binding and biological activity at another melanocortin receptor available in out laboratory, the human MC4-R stably expressed in HEK 293 cells. On this receptors, [D-Trp^7]α-MSH, which was a antagonist at MC3-R, showed slightly higher affinity than α-MSH, but only 15% of its activity (Table 4.3). Nevertheless, it was a full agonist at this receptor. [L-Trp^7] showed lower affinity (34%) and activity (5.3%) than α-MSH.

MC4-293 at agonist	No	Binding (Kd)	c-AMP (EC_{50})
α-MSH	1	1.38×10^{-5} $\pm 4.69 \times 10^{-6}$ n=3	8.05×10^{-9} $\pm 3.25 \times 10^{-9}$ n=3
[Nle^4,D-Phe^7] α-MSH	2	5.08×10^{-7} $\pm 1.24 \times 10^{-7}$ n=3	n.d.
[L-Trp^7] α-MSH	3	4.08×10^{-5} $\pm 6.92 \times 10^{-6}$ n=2	1.52×10^{-7} $\pm 2.34 \times 10^{-8}$ n=3
[D-Trp^7] α-MSH	4	4.53×10^{-6} $\pm 4.04 \times 10^{-7}$ n=3	5.23×10^{-8} $\pm 2.34 \times 10^{-8}$ n=3

Table 4.3.A: Molar dissociation constants, EC_{50} values, and their standard deviation for 7 position at MC4-293 cell. n.d=not determined (Statistics data, appendix 1.14 & 1.15).

MC4-293	No	Kd	EC_{50}
α-MSH	1	1	1
[Nle^4,D-Phe^7] α-MSH	2	27	nd
[L-Trp^7] α-MSH	3	0.34	0.053
[D-Trp^7] α-MSH	4	3.05	0.154

Table 4.3B: Binding affinity and biological activity of [L/D-Trp^7]α-MSH at MC4 receptor relative to α-MSH. n.d=not determined.

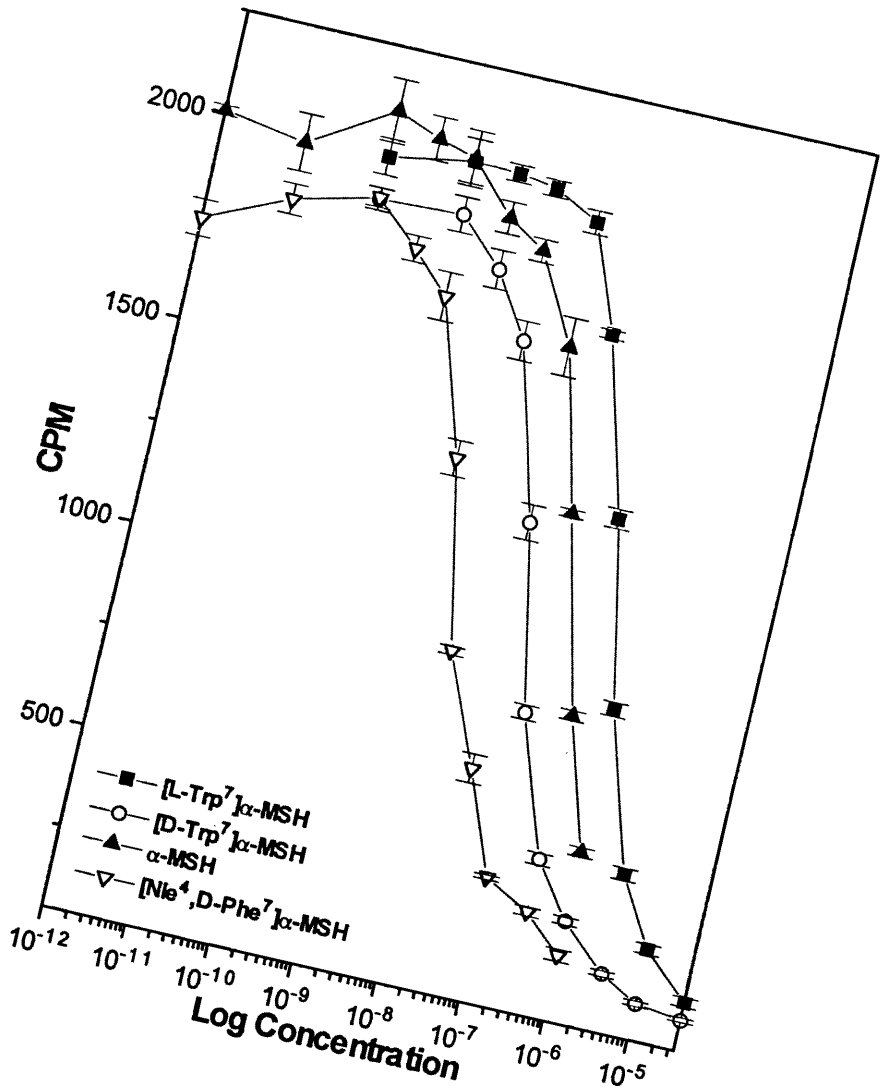


Figure 4.5A: Displacement of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH from the MC4-R.

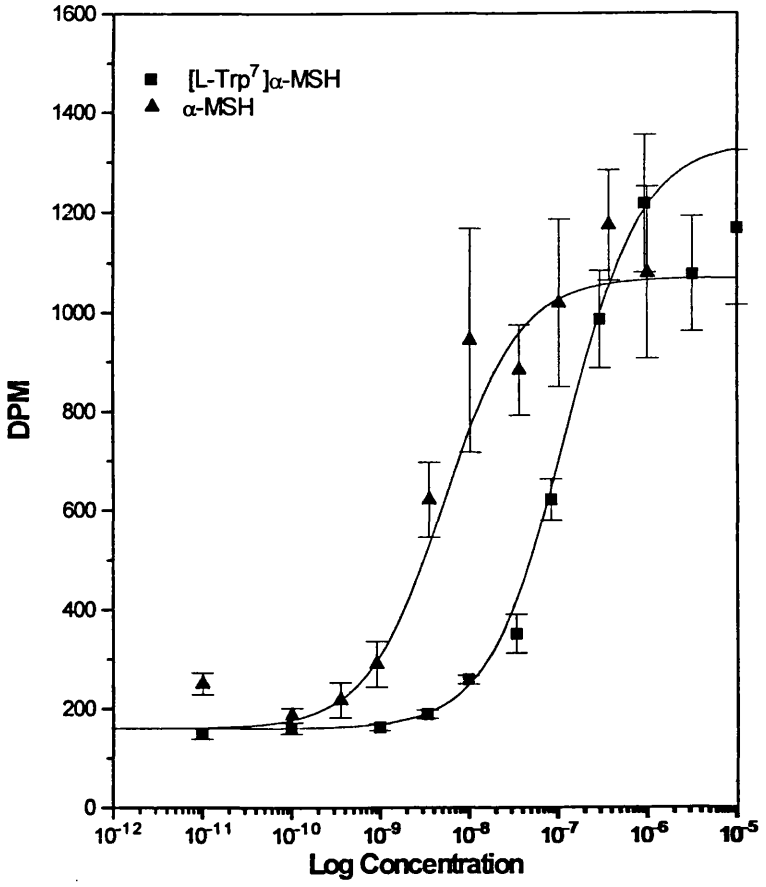


Figure 4.5B: Stimulation of cAMP production at MC4-R.

4.3. Discussion

[L/D-Tyr^7]- and [L/D-Trp^7]α-MSH were synthesised to probe for the importance of position 7 for interaction with MC-R subtypes. Those particular substitutions were chosen because they retained the aromatic structure of the residue with a benzene and an indole moiety, respectively. Replacement of [Phe^7] with a non-aromatic amino acid, alanine, had been demonstrated to lead to a substantial loss of affinity at both MC1-R and MC3-R[134,135]. Introduction of a D amino acid [D-Phe^7] had previously been shown to protect the peptide from chemical and enzymatic degradation[152], thereby leading to a peptide with higher potency and prolonged activity. Thus, the both L- and D-amino acid substitutions were examined in this position.

4.3.1. MC1 Receptor

At the MC1-R, [D-Tyr^7] and [D-Trp^7] peptides are more potent with regard to affinity and activity than are [L-Tyr^7] or [L-Trp^7] analogues. This agreed with Wilkes *et al.*[195] who found that the D-Isomers were more potent than the L-forms in both the frog and lizard skin assay. However, they showed still lower potency than α-MSH except for [D-Tyr^7]α-MSH, which demonstrated a similar affinity and activity to α-MSH. In a computer modelling study by software[190], it has been demonstrated that [D-Phe^7] residue required the minimum energy among central message, Glu-His-Phe-Arg-Trp, and minimum energy leads to higher affinity[189]. It indicated that the D-Phe isomer might have the highest affinity among other substitutions in this position to bind the receptor. Therefore, peptides substituted with D-isomers in position 7,may induce tighter binding to MC1-R, leading to a

higher and sustained, activation of cAMP production than those substituted with L-isomers.

4.3.2. MC3 Receptor

At the MC3-R, as at the MC1-R, substitution with L-amino acids led to peptides with lower affinity than those substituted with their D-isomers. [D-Tyr^7]α-MSH displayed properties similar to those of α-MSH itself. This was also observed at MC1-R. Tyrosine is structurally closer to phenylalanine than tryptophan, and this could be explained this phenomenon. However, despite the introduction of a D-residue this peptide did reach neither affinity nor activity of [Nle^4,D-Phe^7]α-MSH indicating that the phenylalanine residue is nevertheless energetically favoured. However, replacement of phenylalanine with D- or L-tryptophan gave peptides with properties different from the others. While [L-Trp^7]α-MSH displayed low affinity (5% of α-MSH) and was biologically inactive at the concentrations tested, it was found that [D-Trp^7]α-MSH had an affinity similar to α-MSH but had no biological activity. However, it was able to inhibit the activity of α-MSH (at 10^{-8}M concentration) in a dose-dependent manner. This peptide was later shown to exhibit full agonist activity the MC4-R and it is therefore the first melanocortin antagonist that can distinguish not only between MC3-R and MC1-R, but also MC3-R and MC4-R. It might therefore prove a useful tool in the elucidation of the physiological role of the MC3-R which remains to be identified. In the earlier study by Aden *et al.*[158] [Ala^6]α-MSH$_{4-10}$ resulted in a peptide that antagonised the

melanocortin MC3-R and MC5-R but it was less potent at MC4-R, but was still able to act as an antagonist of α-MSH.

4.3.3. MC4 Receptor

α-MSH had lower affinity and activity with MC4-R than that with MC1-R, but [Nle^4,D-Phe^7]α-MSH still showed higher affinity than α-MSH. Thus, the D-configuration remained more potent than L-configuration; this could also be observed for peptides substituted with [L-Trp^7] and [D-Trp^7].

Both [L-Trp^7] and [D-Trp^7] were relatively more active at the MC4-R than at MC1-R. The activity of [L-Trp^7]α-MSH at MC4-R were 34% and 5% and at MC1-R were 0.1% and 0.13%, respectively. The affinity and activity of [D-Trp^7]α-MSH relative to α-MSH at MC4 -R were 3 and 0.15, and at MC1-R were 0.43 and 0.17, respectively. Peptides substituted with [D- or L-Tyr^7] have not been tested at MC4-R. Other peptides substituted with bulky aromatic groups in position 7 have been shown to be antagonists of the MC4-R[159]. However, contrary to the MC3-R, substitution of Phe with tryptophan alone was not sufficient to transform this peptide into an antagonist at the MC4-R.

4.3.4. Summary

Replacement of phenylalanine in position 7 of α-MSH with its D-isomer was long been known to increase both stability and activity of the peptide, and has led to the synthesis of the highly potent analogue [Nle^4,D-Phe^7]α-MSH[152]. It has been

postulated that the [D-Phe^7] substitution leads to minimum energy configuration within the central α-MSH ' core sequence', His-Phe-Arg-Trp, thus causing high affinity binding[189,190]. Likewise, it has been suggested that the D-isomer might lead to 'irreversible' binding at the MC1-R and , thus, to prolonged biological activity[26,144].

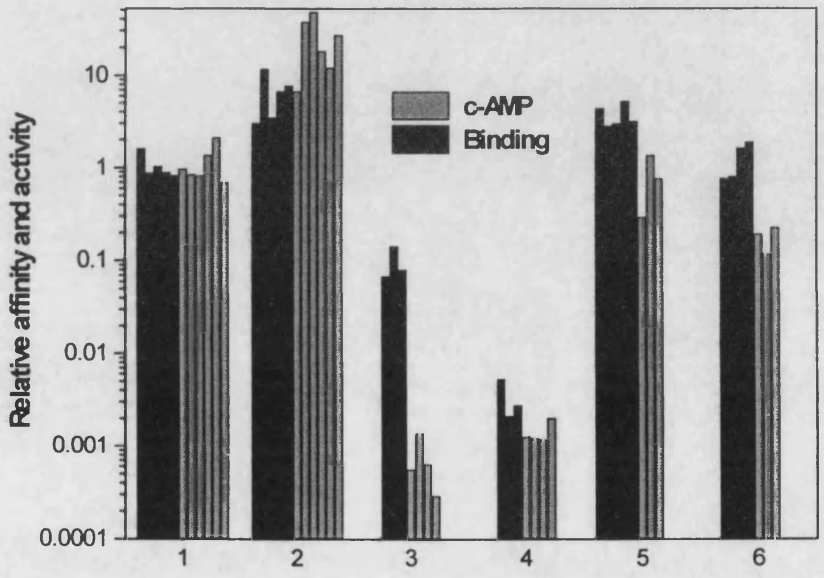


Figure 4.6A: Relative affinity or activity 7 position of α-MSH at MC1-R (log scale). Individual bars represent single experiments. Black = Affinity, Grey = Activity. Refer to Table 4.1 for compound number.

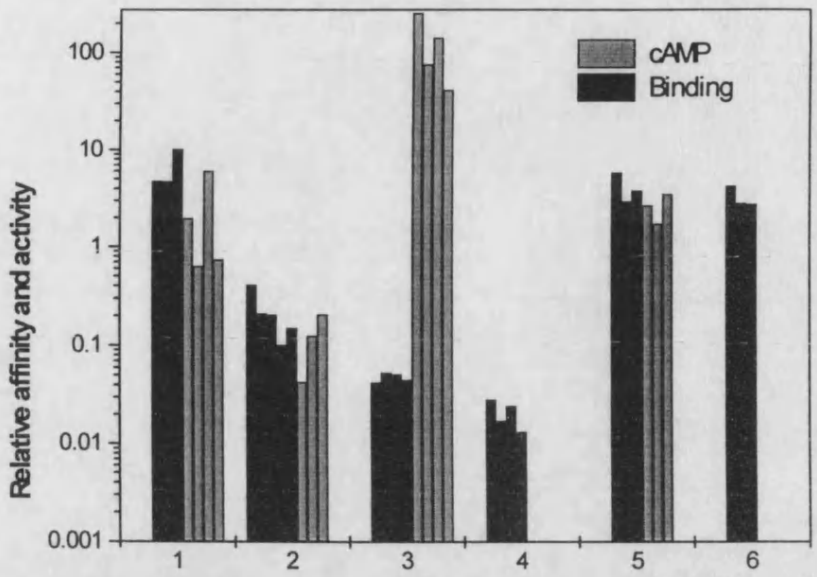


Figure 4.6B: Relative affinity or activity 7 position of α-MSH at MC3-R (log scale). Individual bars represent single experiments. Black =Affinity, Grey = Activity. Refer to Table 4.2 for compound number.

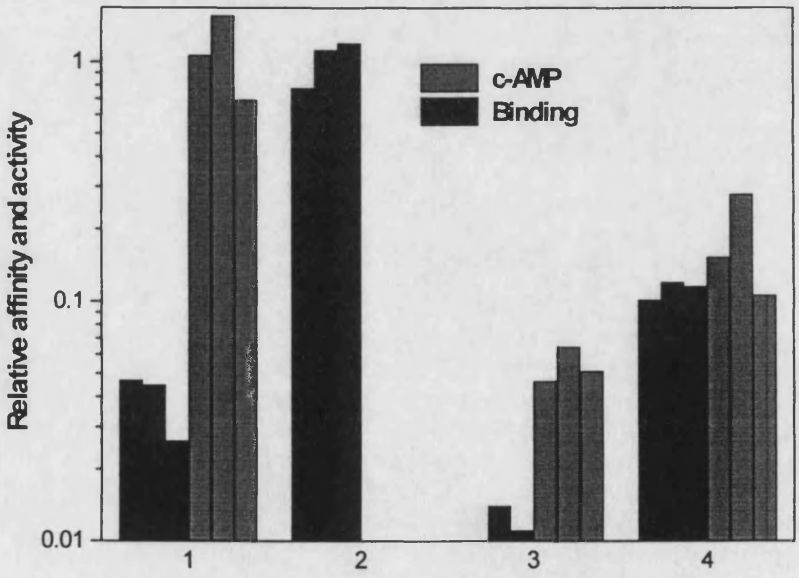


Figure 4.6C: Relative affinity or activity 7 position of α-MSH at MC4-R (log scale). Individual bars represent single experiments. Black =Affinity, Grey = Activity. Refer to Table 4.3 for compound number.

Chapter 5: Activity of Cyclic Peptides at MC1 Receptor

5.1. Introduction

Since linear peptides can adopt a multitude of conformational, states restriction of the conformational flexibility is required for studying the 'receptor-specific' conformation of the peptide. The practical way to obtain semi-rigid analogues of peptides is by cyclisation of suitable linear derivatives[39]. Until recently, most structure-function studies were based on individual amino acid substitutions or deletions in α-MSH and /or shorter linear fragments. Structure-activity relationships with these linear analogous showed the importance of stereochemistry of the amino acid in position 7 to biological potency and prolongation of the melanotropic effect[23], and it was suggested that this might be related to specific conformational effects. Since linear peptides are flexible, interpretation of conformational data on linear D-Phe[7] analogues is difficult[192].

Sawyer *et al.*[148] reported that a β-turn or other peptide chain-reversal region within the central active site, His-Phe-Arg-Trp, of α-MSH might be important in the biologically active conformation of the hormone. Thus, the preparation of a series of highly potent, cyclic, conformationally restricted analogous, cyclized by the pseudoisosteric substitution of cysteine in the 4 and 10 positions for Met[4] and Gly[10] [139,142,148] was based on conformational considerations. [Cys[4],Cys[10]]α-MSH a disulphide-bridged analogue, with conformational restriction compatible with a β-turn conformation, was

the first cyclic α-MSH analogue described[148]. Since cyclisation leads to considerable restriction of conformational flexibility of the peptide backbone and, to a lesser degree, the side chains, a critical examination of these cyclic analogues can provide an important insight into the possible three-dimensional, biologically active structure of α-MSH. Other advantages of cyclic conformationally restricted analogues versus linear derivatives of the native hormone can include either increased potency by stabilisation of the bioactive conformer or decreased enzymatic degradation due to conformational constraints[148].

[Cys^4,Cys^{10}]α-MSH was reported to possess potency about 10000-fold higher than α-MSH in frog skin (*Rana Pipiens*)[139,148]. However, several studies[39,142,149] with [Cys^4,Cys^{10}]α-MSH revealed a much lower potency for this peptide. Cody *et al.*[142] revised their earlier reports to say that [Cys^4,Cys^{10}]α-MSH had about 10-fold higher activity only in the frog skin assay whereas in the other systems of the activity [Cys^4,Cys^{10}]α-MSH was similar to α-MSH. As to the influence of C-terminal tripeptide[142], Lys-Pro-Val-NH_2, it was found that Val[13] was not important, but there was a drastic loss of activity following the loss of Pro[12], with a less dramatic loss after subsequent omission of Lys[11].

The cyclic lactam analogues Ac-[Nle^4,D-Orn^5,Glu^8]α-MSH_{4-11}-NH_2 and Ac-[Nle^4,D-Orn^5,D-Phe^7,Glu^8]α-MSH_{4-11}-NH_2 have a 17-membered ring[187]. The L-Phe cyclic analogue was slightly more potent than the linear Ac-[Nle^4]α-MSH_{4-11}-NH_2 and exhibited prolonged melanotropic bioactivity in the frog skin bioassay but was 100-fold less potent in the lizard bioassay. The D-Phe cyclic analogue was less active than the L-Phe in both assays.

The cyclic lactam analogues, Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$ and Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10},Gly^{11}]α-MSH$_{4-13}$-NH$_2$, were found to possess the highest potencies among the cyclic heptapeptides in lizard skin and tyrosinase assays[150,151]. The ring size is 23 atoms. The influence of N-terminal amino acids on affinity or activity has not been tested, but it has been attempted to add fatty acids to the N-terminal of the Ac-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$ analogue which displayed 10-100-fold the biological activity of α-MSH in the Cloudman S91 tyrosinase assay[188].The shorter conjugates of hexanoic and decanoic acid were as potent as α-MSH in the lizard skin bioassay, whereas the longer myristoyl and palmitoyl analogues were about 100-fold less potent[188]. Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$ has 90 times greater potency than α-MSH in lizard skin and 100-fold in tyrosinase assay[150,151].

The object of this study is to design of a semi-rigid synthetic analogue of α-MSH in order to understand its physical and biological properties and their possible relationship to the biologically active conformation of the peptide. Here, we included the C-terminal, Lys-Pro-Val-NH$_2$ into our study to test whether it preferably affects the conformational properties of the semi-rigid Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$-NH$_2$, therefore, we synthesised [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-13}$-NH$_2$ analogues to examine binding to the cloned murine B16 MC1-R, and their ability to stimulate c-AMP production.

5.2. Results

All peptides were able to inhibit completely the binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to murine B16 melanoma cells and exhibit full activity at different concentration (Table 5.1). The relative potency of these compounds to α-MSH are given in Table 5.2. The result of competitive binding assay with [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH for α-MSH, and cyclic peptides are shown in Figure 5.1. The ability to generate cAMP in response to melanocortin peptides is illustrated in Figure 5.2 and 5.3 and the relative affinity and activity are shown in Figure 5.4.

The cyclic compounds have at least 10-fold higher affinity and activity than α-MSH except for [$\overline{\text{Asp}^5,\text{D-Phe}^7,\text{Lys}^{10}}$]α-MSH$_{5\text{-}13}$ (Table 5.1), which lacks Nle^4 and shows the same affinity but slightly higher activity (4.4-fold) than α-MSH. The compound **3** has same affinity and about 10-fold higher activity than α-MSH. The compound **5** has highest affinity and activity of the peptides tested which was increased by a factor of 17.6 and 25.6, respectively, when compared to α-MSH.

B16	No	Binding (k_d)	c-AMP (EC_{50})
α-MSH	1	1.68×10^{-8} $\pm 3.88 \times 10^{-9}$ n=5	2.74×10^{-9} $\pm 9.56 \times 10^{-10}$ n=6
[Nle^4,D-Phe^7]α-MSH	2	3.27×10^{-9} $\pm 1.78 \times 10^{-9}$ n=5	1.67×10^{-10} $\pm 1.30 \times 10^{-10}$ n=6
[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-13}$	3	1.80×10^{-9} $\pm 3.57 \times 10^{-10}$ n=3	3.00×10^{-10} $\pm 2.78 \times 10^{-10}$ n=4
[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-13}$	4	1.72×10^{-8} $\pm 6.79 \times 10^{-9}$ n=4	6.24×10^{-10} $\pm 3.71 \times 10^{-10}$ n=4
[Nle^4,Asp^5, D-Phe^7, Lys^{10}]α-MSH$_{1-13}$	5	9.56×10^{-10} $\pm 2.99 \times 10^{-10}$ n=4	1.7×10^{-10} $\pm 5.64 \times 10^{-11}$ n=4

Table 5.1. Binding affinity (expressed as dissociation constant) and biological affinity (expressed as EC_{50}) of [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-13}$ analogues at MC1 receptors. Errors represent the standard deviation determined from replicate experiments (Statistics data, Appendix 1.116).

B16	No	Binding	c-AMP
α-MSH	1	1.0	1.0
[Nle^4,D-Phe^7]α-MSH	2	5.14	16.4
[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-13}$	3	9.3	9.13
[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-13}$	4	0.98	4.4
[Nle^4, Asp^5, D-Phe^7, Lys^{10}]α-MSH$_{1-13}$	5	17.6	25.6

Table 5.2. Binding affinity and biological activity of [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH analogues at MC1 receptors relative to α-MSH$_{4-13}$.

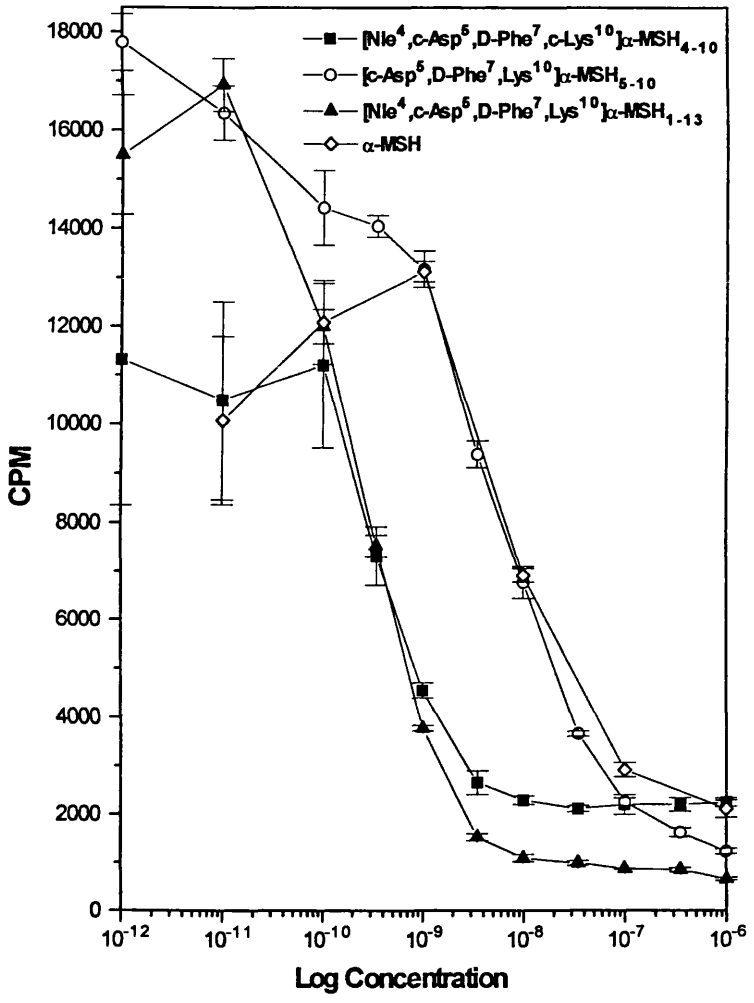


Figure 5.1 Displacement of [^{125}-I-Tyr^2,Nle^4,D-Phe^7] α-MSH from the MC1 receptors by [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH analogues.

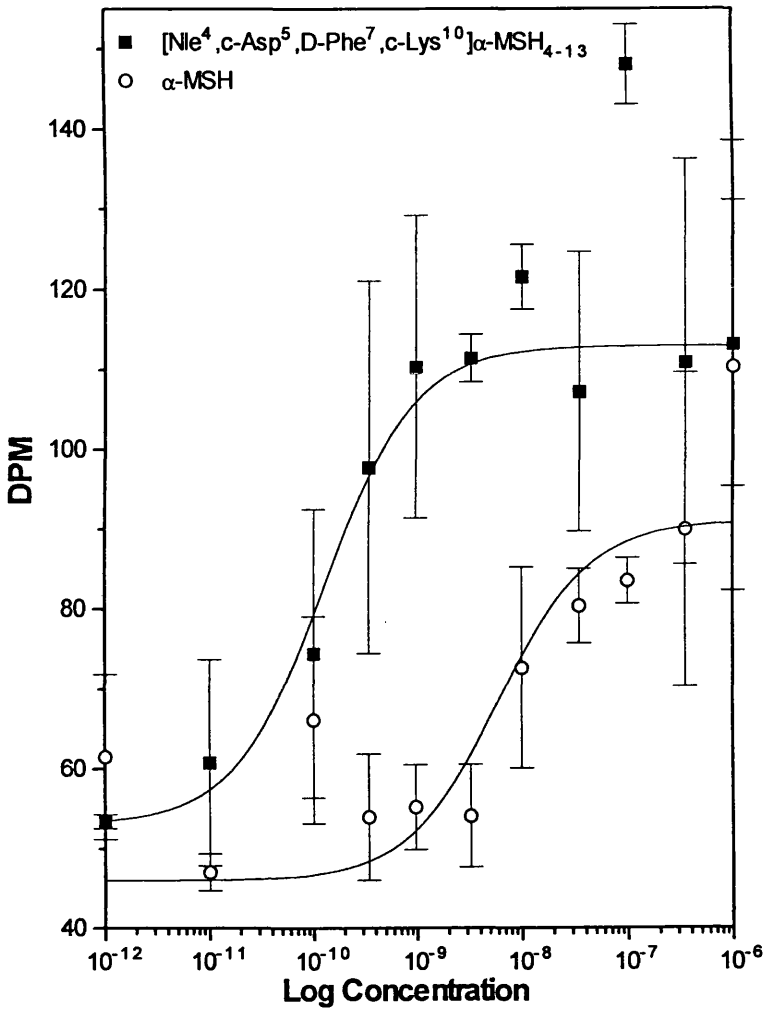


Figure5.2. Stimulation of cAMP production at MC1 receptors by [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-13}$ and α-MSH.

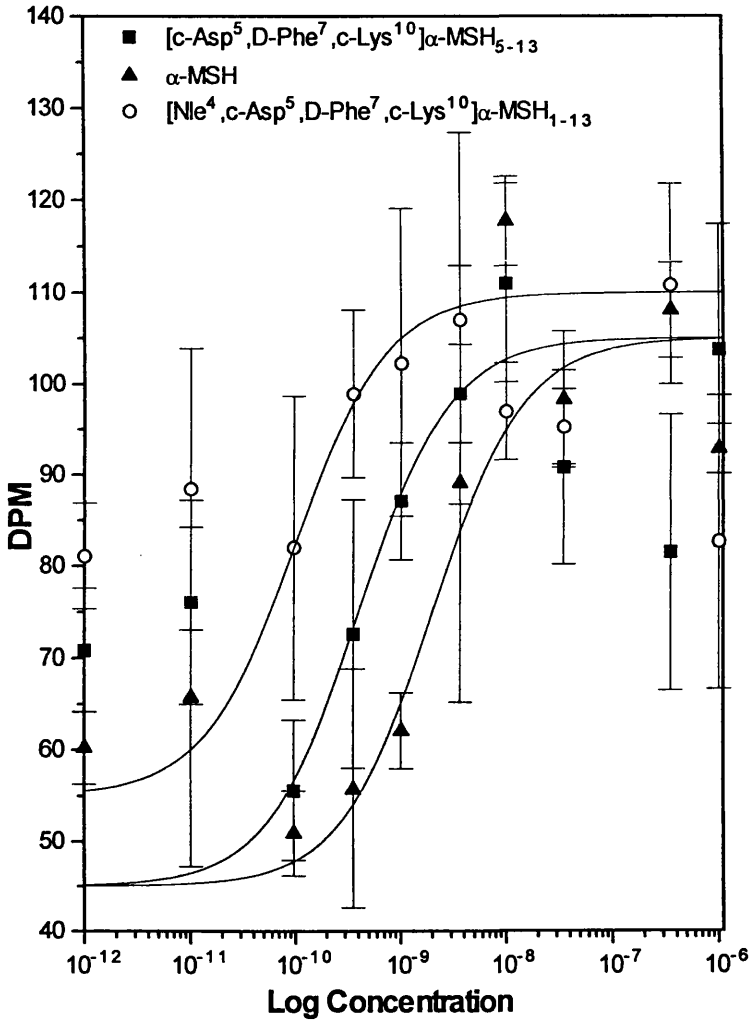


Figure 5.3 Stimulation of cAMP production at MC1 receptors by [Nle^4,$\overline{\text{Asp}^5,\text{D-Phe}^7,\text{Lys}^{10}}$]α-MSH$_{1\text{-}13}$, [Nle^4,$\overline{\text{Asp}^5,\text{D-Phe}^7,\text{Lys}^{10}}$]α-MSH$_{5\text{-}13}$ and α-MSH.

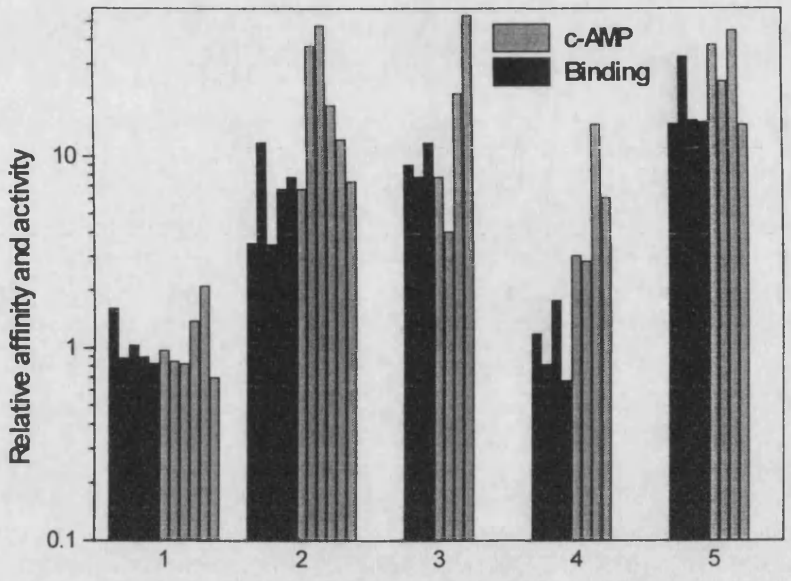


Figure 5.4. Relative affinity and activity of [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH analogues and α-MSH. Bars represent single experiments

5.3. Discussion

In general, the cyclic [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$ analogues are reported to be more potent than [Nle^4,D-Phe^7]α-MSH[152,153] compared to α-MSH. It has been proposed that [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$ has relative potency 90-fold in the lizard skin bioassay and that 100-fold in tyrosinase assay[150,151]. In our study a peptide that included the C-terminal tripeptide, Lys-Pro-Val-NH$_2$, [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-13}$ (Table 5.1 and 5.2) is 9 times more potent in affinity and activity than α-MSH at murine B16 melanoma cells, giving a similar result to [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$ at the human MC1-R[152,153]. We did not synthesise the [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$ to compare result. Compared to α-MSH, our data suggested that the C-terminal was not as important for increasing the activity in [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$ assay. Yet, this requires to synthesis more cyclic peptides to be certain.

Analogue **4** (without Nle^4) showed the same affinity as α-MSH but was slightly more active than α-MSH, indicating that [Nle^4] is important for activity. Nle is pseudoisosteric to Met, but does not contain sulphur which is easily oxidised with subsequent loss of potency in α-MSH[191]. Studies with linear α-MSH fragments have already revealed the relative importance of this residue for affinity and activity of the peptide[39,140].

The peptide which included N-terminal, Ser-Tyr-Ser- had the highest affinity and activity compared to others in this study, suggesting that the N-terminal was important to enhance the activity of [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$ analogues. Compound

5, had 17-fold higher affinity and about 25-fold higher activity than α-MSH. That the cyclic peptide, **5** , has a higher affinity and activity than [Nle^4,D-Phe^7]α-MSH indicated that restricted analogues versus linear derivatives might increase potency by stabilisation of the bioactive conformer or reduce enzymatic degradation due to conformational constraints as has been suggested previously[148].

Modern computer technology has made it possible to extend theoretical conformational studies of peptides to examine their molecular dynamics and internal energetics, and energy minimisation of peptide structure has been used recently[190]. High affinity ligands in general have low conformational energies, and high conformational energies correspond to low affinity ligands[189]. D-Phe^7, Asp^5 substitutions have shown the lowest energy in molecular modelling[190], indicating that D-Phe^7, Asp^5 analogues might have high affinity toward melanocortin receptors. This could explain why the affinity of [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{1\text{-}13}$ is higher than [Nle^4,D-Phe^7]α-MSH.

5.4. Future Studies of Cyclic Peptides

Due to their stronger activity compared to α-MSH at MC1-R, [Nle^4,D-Phe^7]α-MSH and [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4\text{-}10}$ have been tested for clinical use as tanning agents. A great deal more work needs to be done to evaluate the structural-activity relationships between cyclic analogues and melanocortin receptors. So far, the cyclic peptides have only been examined at MC1-R, so it would be worthwhile to study effects of melanocortin peptides at different receptors.

Efforts to understand the relationships of conformational to biological activity of α-MSH utilising physical and molecular modelling methods are still in an elementary state. The studiy[192] reported indicate that an aqueous solution of α-MSH and its analogous can assume energetically preferred conformations, but there is still much to be done to determine with greater certainty and clarity the precise nature of these conformational preferences. Also, it remains questionable whether an aqueous solution of the peptide reflects the physiological conditions at the moment of receptor binding.

Linear and cyclic analogues of α-MSH have been developed which have receptor selectivity, potency, and exceptionally prolonged *in vitro* and *in vivo* biological activities. These findings offer considerable incentive to develop conformational structure models which might explain these biological results, and to test these models with appropriate conformational constraints consistent with the biological and biophysical data. It can be anticipated that a combination of design and synthesis based on conformational and modelling molecular mechanics, and molecular dynamics approaches, and biophysical studies on constrained analogous, will provide significant new insights into α-MSH conformation-activity relationship.

Chapter 6: Discussion

The work in this thesis is concerned with elucidating the structure-activity relationships of melanocortin peptides. A series of peptides were tested at the melanocortin MC1 and MC3 receptors with the aim of designing receptor-specific ligands. While this was originally undertaken with a view to the therapeutic potential melanocortins in the treatment of melanoma, the recent discovery of the function of the melanocortin MC4-R opens a new field of potential applications for melanocortin peptides.

In this chapter, the results presented in chapters 3, 4 and 5 are summarised and put into context with the current state of melanocortin research.

6.1. Origin of α- MSH

α-MSH and ACTH are a group of related peptides containing the typical core sequence, His-Phe-Arg-Trp, and are derived from a common precursor, proopiomelanocortin (POMC). They occur in the pituitary, some brain regions, and also in several peripheral tissues. Adult humans do not have a pituitary intermediate lobe, and thus have very little α-MSH in the serum. In addition, most of β-LPH is not processed into β-MSH, so that β-LPH and ACTH are the two predominant melanocortin in man, while α-MSH is the predominant melanocortin in most other species[198]. Although POMC appears to be processed differently in various brain regions. The highest concentration of α-MSH in the brain is in the hypothalamus. Also of interest is the presence in some species of desacetyl-α-

MSH, which has a slightly different pharmacological profile to α-MSH, and will not exhibit all its functions[201].

6.2. Identification of a Family of MSH Receptors

A family of five melanocortin receptor subtypes has been identified to date. Each member of the melanocortin receptor family has a distinct tissue distribution. All are G-protein-coupled receptors that activate adenylyl cyclase and bind melanocortins. To date the MC1-R has been reported to be expressed in melanocytes, melanoma tissue[15,16], and macrophages[126,127], while the MC2-R is expressed in all the adrenal cortex[15]. The MC3-R and MC4-R are predominantly expressed in brain. The MC3-R has a restricted distribution, with the greatest density in hypothalamic cells[62]. In contrast, MC4-R is more widely expressed than MC3-R and is found in multiple sites in almost every brain region[197]. In addition to its expression in brain, the MC3-R was found in placenta and stomach, pancreas[17]. The MC5-R was found in a broad spectrum of tissues including skin, brain, skeletal, muscle, lung, spleen thymus, bone marrow, testis, ovary, uterus, and adrenal gland[171-177].

The melanocortin receptors have different pharmacological properties, as predicted by binding and c-AMP assay. The MC1-R binds and is stimulated well by α-MSH, while MC2-R preferentially binds ACTH. The MC3-R appeared to respond relatively well to γ-MSH; while MC4-R had a higher affinity for β-MSH. MC5-R has been expressed in four species and each has a slightly different pharmacological profile among species.

6.3. Selective Ligands for Melanocortin Receptors

6.3.1. C-Terminal Substitution

At murine MC1-R, the C-terminal amino acids play an important role in differentiating between MC1-R and MC3-R. At the murine MC1-R, proline 12 (numbering with reference to α-MSH) appears to be important residue for recognition. Replacement of this position with any residue was expected to influence the properties of α-MSH and γ-MSH analogues as long as L-phenylalanine was present in position 7.

The experimental work carried out here suggested at MC1-R, all analogues of [Nle^4,D-Phe^7]α-MSH bound with equal affinity and exhibited the same biological activities irrespective of their substitution at position 12, but the α-MSH analogue, [Phe^{12}]α-MSH, showed a significant reduction of both affinity and activity. All γ-MSH peptides showed very low affinity and activity at the MC1-R. This was expected from the literature[53,186]. Of the γ-MSH peptides tested, only [Nle^3,Pro^{11}]γ_1-MSH was more potent than γ_1-MSH. Substitutions with multiple alanine within the C-terminal had lower activity than α-MSH. Therefore, it was concluded that position 12 is important in the interaction of α-MSH with murine MC1-R.

At the MC3-R, C-terminal substitutions to α-MSH did not result in significant changes on the potency. All analogues of [Nle^4,D-Phe^7]α-MSH showed higher affinity and increased biological activity, suggesting that D-Phe^7 analogues bound more stably to the receptor. The α-MSH analogue, [Phe^{12}]α-MSH, had its activity

similar to α-MSH. γ-MSH peptides bound to the rat MC3-R with reduced affinity compared to α-MSH, but their biological activity was in the same range as that of α-MSH. All γ-MSH analogues except [Nle^3,Pro^{11}]γ_1-MSH showed increased selectivity for the MC3-R. [Nle^3,Pro^{11}]γ_1-MSH was not more selective for the MC3-R than α-MSH. The observation of increased efficacy and selectivity of γ-MSH peptides at the MC3-R supported the suggestion originally made[62], that the MC3-R may respond to γ-MSH.

It was concluded that substitutions at the C-terminal of α-MSH have greater impact at MC1-R than MC3-R as long as position 7 remains in the L-configuration. Thus, the C-terminal might be the way to distinguish between the activity of linear peptide at the MC1-R and MC3-R.

6.3.2. Antagonist

It has been known for sometime that replacement of phenylalanine in position 7 of α-MSH with its D-isomer increases both stability and activity of the peptide, and this has led to synthesis of the highly potent analogue [Nle^4,D-Phe^7]α-MSH[152]. Thus, a series of substitutions at 7 were investigated. In general, substitution with L-amino acids led to peptides with lower affinity than those substituted with their D-isomers at both MC1-R and MC3-R. These peptides had lower affinity than α-MSH except for [D-Tyr^7]α-MSH, which had a similar affinity and activity to α-MSH. Tyrosine is structurally closer to phenylalanine than tryptophan. [D-Trp^7]α-MSH had full agonist activity at the MC1-R and MC4-R but antagonised the action of α-MSH with an IC_{50} of 1.95 ± 0.628 x 10^{-7}M. Therefore, [D-Trp^7]α-

MSH may be a lead compound for development of selective ligands.

Hruby *et al.*[159] reported that substitution in the position 7 of Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$ can produce potent and selective antagonists for melanocortin receptors. [D-Trp^7]α-MSH appears to be a selective antagonist for MC3-R.

6.3.3. Cyclic Peptides

In this study, addition of the C-terminal in cyclic peptides did not appear to be as important at MC1-R compared to studies of linear peptides. [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-13}$ showed similar affinity and activity at murine B16 melanoma cells compared to [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$ at human MC1-R. In contrast, addition of the N-terminal to produce 1-13 analogue led to higher affinity and activity compared to others in this study, suggesting that the N-terminal was important to enhance the activity of cyclic peptides based on [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-10}$. Such as [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{1-13}$ had 17-fold higher affinity and 25-fold higher activity than α-MSH. These peptides with further study at need to be compared with the Hruby *et al.* compared MT-II.

6.4. Agouti and Obesity

Mammalian melanocytes can produce two types of melanin, eumelanin (black or brown) and phaeomelanin (red or yellow). The pathways are controlled by the products of two classical coat-colour controlling gene loci, extension and agouti.

The mouse agouti gene encoded an 131 amino acid agouti protein that instructs hair follicle melanocytes to switch from black to yellow pigment. When agouti is overexpressed in yellow mutant mice, it induces obesity and diabetes. Lu *et al.*[85] have shown that the agouti protein is an antagonist at the MC1-R. This explains the effects of agouti on pigmentation, however, it remains possible that agouti peptide has other physiological actions, either via melanocortin receptor or independent of melanocortin receptor. Hunt *et al.*[93] have demonstrated that the agouti protein can act independently of MSH to inhibit melanogenesis. Intracellular free Ca^{2+} concentration is elevated in skeletal muscle of yellow mice compared with nonagouti mice and agouti peptide induces an increase in intracellular Ca^{2+} in isolated skeletal muscle from wild type mice[92].

Recently, activity has focused on the potential role of melanocortin peptides in the yellow obese mouse since the discovery that agouti peptide not only antagonises α-MSH at the MC1-R but also antagonises α-MSH at the MC4-R[85]. Lu *et al.*[85] expressed MSH receptors in 293 cells and assayed the ability of agouti to inhibit generation of adenylyl cyclase activity by α-MSH. The results demonstrated that agouti was a high affinity antagonist of both the MC1-R and MC4-R *in vitro*, but not of the MC3-R and MC5-R. This suggests that antagonism of the MC4-R might be crucial in regulating weight. Injecting synthetic peptides mimicking melanocortin into the brain of both normal and obesity mutant mice, Fan *et al.*[89] found that the molecules bind to MC4-R and suppress feeding, even when the mice are given neuropeptide Y, potent appetite-stimulating neurotransmiter, to increase the animal's appetites during the night or after a prior fast. Huszar *et al.*[199] show that depletion of the MC4-R produces an obesity syndrome, thus defining a

novel function for the MC4-R in the regulation of energy balance. The synthetic α-MSH antagonist used by these authors was SHU9119, which is a potent antagonist at MC4-R, but full agonist at MC1-R thus confirming the involvement of the MC4-R in the control of feeding behaviour.

6.5. Future Work

The effect of melanocortin peptides, notably α-MSH, on melanogenesis in melanocytes appears well established, but their role in melanocyte and melanoma cell proliferation is less clear and remains to be investigated in more detail. The presence of MC1-R on melanocytes and more importance melanoma cells might in the future be exploited for diagnostic and/ or therapeutic purposes. To this end, selective, high-affinity ligands of MC1-R are needed.

The role of the MC3-R, as that of the MC5-R, remain unclear. While the MC3-R shows a comparative preference for γ-MSH peptides, it seems unlikely that it participates in mediating the cardiovascular actions of these peptides. The discovery of a selective MC3-R antagonist (described in chapter 4) might help in elucidating the biological roles of this receptor. The antagonist, [D-Trp^7]α-MSH, is of low affinity, but acts as an agonist at MC1-R and MC4-R. It is predicted that will be chemical stable under *in vitro* and *in vivo* conditions due to the D-amino acid in position 7. It could therefore be used directly, or might serve as a lead compound in the search for selective antagonists with higher affinity.

The discovery that MC4-R participates in the control of body weight has led to renewed interest in melanocortin receptors and MSH peptides. The development of

MC4-R antagonist that can be used in weight-reducing therapies is potentially of great therapeutic and commercial interest. This also opens the field for the design of non-peptide antagonists, which would have an advantage over peptide antagonist, as they are generally less expensive to produce and have better pharmacokinetic properties.

There are several physiological properties of melanocortin peptides that cannot be explained by their actions at the receptors already known, the existence of future melanocortin receptors seems likely.

The mode of interaction of melanocortin receptors with their ligands remains to be studied. For the MC1-R, attempts have been made by point-mutation studies, to identify the binding site of the receptor. These studies need to be continued and also carried out with other melanocortin receptors. They would greatly helped by the existence of antibodies, but to date, the only one described in the literature is a polyclonal MC1-R antibody.

Finally, the existence of high-affinity cyclic agonists and antagonists will enable conformational and molecular modelling studies to elucidate the interaction of MSH peptides with their receptor.

6.6. Final Conclusions

As with many pieces of scientific work, and particularly with those strictly limited by time or resources, the experiments and results reported in this thesis suffer shortcomings that might be rectified if further time were made available or if the project were to be started again with the benefit of hindsight. Two principal issues are as follows:

- If the work were being repeated it might increase the value of the results if

experiments were performed using human cell lines and receptors instead of, or in addition to mouse cell lines and receptors. Although cell lines containing a range of human MSH receptors were not available at the time this work was started, recently the human MSH-R has become available transfected into Cos7 cells or into the murine L cell line.

- In addition to the *in vitro* experiments performed on cultured cells, it would have been useful, and it would be valuable in the future, if some experiments were performed *in vivo* in animal models. In particular it would be interesting to test the compound [D-Trp^7]α-MSH, shown in this thesis to be a selective antagonist of rat MC3-R, in behavioural studies and possibly also using *in situ* hybridisation studies in the rat.

Looking back at the thesis as a whole there are some sections of results which rather than provide answers to questions, instead pose further questions.

A fundamental issue that is the basis of any study looking at a range of analogues of a pharmacologically active compound, in this case analogues of the peptide α-MSH, is the relationship between binding affinity and biological activity. It is expected that modification of the lead compound will either decrease or increase the binding affinity. If the binding affinity is increased and there is also an increase in biological activity, the new analogue is likely to be a potent agonist. Sometimes what is observed is no change or perhaps an increase in binding affinity but with reduced or perhaps no associated biological activity. In this case the analogue is likely to be an antagonist. If an agonist or an antagonist is selective in its action it is likely to be useful in elucidating the role of the associated receptor.

- In the present work a wide range of modifications to melanocortins have produced changes in binding affinity and biological activity. One set of experiments have produced results that are at present not easily explained, but should be noted as of interest for the future. The measure of biological response used in this thesis has been elevation of c-AMP levels. In a number of experiments, modification of the peptide has resulted in a change in the binding

affinity, in most instances as expected the associated biological activity, as measured by c-AMP levels changes proportionally. In a few cases there is a relative reduction in c-AMP levels, interpreted as the action of an antagonist. However in some cases the change in c-AMP levels is such that it suggests increased biological activity for a given level of binding affinity. Examples of this observation can be seen with compound tested in lanes 6,7 and 8 in Fig 3.7B (page 87) and in lane 1 in Fig. 4,6C (page 132). One interpretation of these results is that they are artifacts associated with the relative sensitivity of the binding assay and the c-AMP assay. While the c-AMP assay is known to be a difficult assay the evidence in this thesis does not indicate any lack of reliability. However it would be interesting in future work, in particular with those compounds giving these anomolous responses, to look at other alternative measures of biological response, perhaps tyrosinase enzyme levels or melanin production where appropriate.

Taking into account the areas above which leave room for improvement and further work, it is worth emphasising the principal results which have arisen out of this thesis and which may act as leads for further work and hopefully may contribute, albeit in a small way, to a greater understanding to the action of melanocortins.

- The main finding in this thesis is the discovery of a selective antagonist for the rat MC3 receptor. The MSH analogue [D-Trp^7]α-MSH is selective for rat MC3-R with an IC_{50} of 1.95 x 10^{-7}M. This is seen most clearly in Figs. 4.3C and 4.4. In relation to this observation, it has been proposed that the [D-Phe^7]α-MSH substitution leads to a minimum energy conformation within the central α-MSH 'core sequence', His-Phe-Arg-Trp, causing high affinity binding. The hypothesis has previously been put forward that the D-isomer in [D-Phe^7]α-MSH might lead to 'irreversible' binding at the MC1-R and this might have relevance to the observations reported here with [D-Trp^7]α-MSH and MC3-R. The discovery that [D-Trp^7] α-MSH is a selective antagonist of rat MC3-R may be of direct use in the elucidation of the biological importance of this receptor

or it may lead to the development of higher affinity antagonists in the future.

Lastly it should be asked if the results reported in this thesis may have any use in clinical treatment of human disease. There are two clinical areas where the development of high affinity agonists or antagonists may be of clinical value.

- Firstly there is increasing evidence of the importance of melanocortins in a number of physiological functions and in associated pathological conditions. These include, in addition to pigmentation disorders, nerve regeneration, behavioural functions e.g mood control, and most recently and importantly functions associated with obesity. The development of a selective agonists and antagonists for example [D-Trp^7]α-MSH may be of benefit to research and eventually treatments in these areas.

- Secondly it has been suggested that one approach to treatment of those cancers not effectively treated by surgery, radiotherapy or current chemotherapy protocols might be treated more effectively by cytotoxic agents if they could be delivered selectively to the target cells. In the case of melanoma, which is a cancer that is often fatal and is increasing in incidence in the UK, there exists the possibility that cytotoxic agents could be targeted to melanoma cells by attaching the cytotoxic agent to a ligand for melanocortin receptors. In this way the cytotoxic agent might be selectively delivered to melanoma cells. A number of difficulties exist in this approach, receptors are likely to found on cells other than the melanoma cells and these cells might also be killed. Also there may be difficulties in delivering enough cytotoxic molecules to each cell, the number of MC receptors per cell is likely to be in the order of 10's of thousands whereas the number of cytotoxic molecules required per cell to effect killing may be orders of magnitude higher. For example it has been estimated that the commonly used cytotoxic agent methotrexate requires approximately 10^6 molecules per cell to kill the cell. In the future the use of more potent cytotoxic agents or perhaps the use of gene therapy agents may mean that smaller numbers of molecules are needed per cell. In this case the development of high affinity MC-R ligands may be useful as targeting agents.

Chapter 7: References

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Appendix 1

Statistical Analysis of the Biological Data

Appendix 1.1: Data of Binding Assay in α-MSH analogues at B-16 Melanoma Cells.

Appendix 1.2: Data of c-AMP in α-MSH analogues at B-16 Melanoma Cells.

Appendix 1.3: Data of Binding Assay in α-MSH analogues at MC3-R.

Appendix 1.4: Data of c-AMP in α-MSH analogues at MC3-R.

Appendix 1.5: Data of binding Assay for Multiple Alanine Substitution Analogues in α-MSH at B-16 Melanoma Cells.

Appendix 1.6: Data of c-AMP for Multiple Alanine Substitution Analogues in α-MSH at B-16 Melanoma Cells.

Appendix 1.7: Data of Binding Assay for Alanine Substitution Analogues in α-MSH at MC3-R.

Appendix 1.8: Data of c-AMP for Alanine Substitution Analogues in α-MSH at MC3-R.

Appendix 1.9: Data of Binding Assay for [L/D-Tyr^7]α-MSH, [L/D-Trp^7]α-MSH in at B-16 Melanoma Cells.

Appendix 1.10: Data of c-AMP for [L/D-Tyr^7]α-MSH,[L/D-Trp^7] α-MSH at B-16 Melanoma Cells.

Appendix 1.11: Data of Binding Assay for [L/D-Tyr^7]α-MSH,[L/D-Trp^7]α-MSH at MC3-R.

Appendix 1.12: Data of c-AMP for [L/D-Tyr^7]α-MSH at MC-3R.

Appendix 1.13: Data of c-AMP Antagonist for [L/D-Trp^7]α-MSH at MC3-R.

Appendix 1.14: Data of Binding Assay for [L/D-Trp^7]α-MSH at MC4-R.

Appendix 1.15: Data of c-AMP for [L/D-Trp^7]α-MSH at MC4-R.

Appendix 1.16: Data of Binding Assay for Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-13}$ at B-16 Melanoma Cells.

Appendix 1.17: Data of c-AMP for Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-13}$ at B-16 Melanoma Cells.

Appendix 1.1: Data of Binding Assay in α-MSH analogues at B-16 Melanoma Cells.

ROW	C1	C2	C3
1	1	1.04	0.03922
2	1	1.89	0.63658
3	1	1.62	0.48243
4	1	1.86	0.62058
5	1	2.01	0.69813
6	2	0.55	-0.59784
7	2	0.14	-1.95193
8	2	0.48	-0.73397
9	2	0.25	-1.39837
10	2	0.21	-1.54178
11	3	0.11	-2.21641
12	3	0.09	-2.41800
13	3	0.10	-2.34237
14	4	0.13	-2.06357
15	4	0.28	-1.26231
16	4	0.16	-1.86433
17	5	0.28	-1.25878
18	5	0.33	-1.10262
19	5	0.31	-1.17441
20	5	0.28	-1.26585
21	5	0.14	-1.95193
22	6	76.10	4.33205
23	6	143.00	4.96284
24	6	199.00	5.29330
25	7	24.80	3.21084
26	7	37.00	3.61092
27	7	95.60	4.56017
28	8	399.00	5.98896
29	8	6380.00	8.76092
30	8	1420.00	7.25841
31	9	635.00	6.45363
32	9	343.00	5.83773
33	9	179.00	5.18739
34	10	2222.00	7.70616
35	10	999.00	6.90675
36	10	819.00	6.70808
37	11	1100.00	7.00307
38	11	1980.00	7.59085
39	11	2790.00	7.93380
40	12	110.00	4.70048
41	12	43.20	3.76584
42	12	21.50	3.06805
43	13	1020.00	6.92756
44	13	764.00	6.63857
45	13	401.00	5.99396
46	14	1920.00	7.56008
47	14	1160.00	7.05618
48	14	1520.00	7.32647

```
MTB > onew c3 c1;
 SUBC> fisher 0.05;
 SUBC> tukey.

 ANALYSIS OF VARIANCE ON C3
 SOURCE      DF         SS         MS          F        p
 C1          13    667.338     51.334     152.67    0.000
 ERROR       34     11.432      0.336
 TOTAL       47    678.771

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
  LEVEL       N       MEAN      STDEV ---------+---------+---------+-------
      1       5     0.4954     0.2669           (*-)
      2       5    -1.2448     0.5682      (*-)
      3       3    -2.3256     0.1018  (-*-)
      4       3    -1.7301     0.4172    (-*-)
      5       5    -1.3507     0.3427      (*-)
      6       3     4.8627     0.4884                      (-*-)
      7       3     3.7940     0.6930                   (-*-)
      8       3     7.3361     1.3876                           (-*-)
      9       3     5.8262     0.6332                 (-*-)
     10       3     7.1070     0.5283                    (-*-)
     11       3     7.5092     0.4707                      (*-)
     12       3     3.8448     0.8191                   (-*-)
     13       3     6.5200     0.4780                       (-*-)
     14       3     7.3142     0.2522                         (-*-)
                                      ---------+---------+---------+-------
 POOLED STDEV =    0.5799            0.0       3.5       7.0
```

1
Tukey's pairwise comparisons
Family error rate = 0.0500
Individual error rate = 0.000987

Critical value = 5.10

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5	6	7
2	0.418 3.063						
3	1.294 4.348	-0.446 2.608					
4	0.698 3.753	-1.042 2.012	-2.303 1.112				
5	0.524 3.169	-1.217 1.429	-2.502 0.552	-1.907 1.148			
6	-5.895 -2.840	-7.635 -4.580	-8.896 -5.481	-8.300 -4.885	-7.741 -4.686		
7	-4.826 -1.771	-6.566 -3.512	-7.827 -4.412	-7.231 -3.817	-6.672 -3.618	-0.639 2.776	
8	-8.368 -5.314	-10.108 -7.054	-11.369 -7.954	-10.774 -7.359	-10.214 -7.160	-4.181 -0.766	-5.250 -1.835
9	-6.858 -3.804	-8.598 -5.544	-9.859 -6.444	-9.264 -5.849	-8.704 -5.650	-2.671 0.744	-3.740 -0.325
10	-8.139 -5.084	-9.879 -6.825	-11.140 -7.725	-10.544 -7.130	-9.985 -6.931	-3.952 -0.537	-5.020 -1.606
11	-8.541 -5.487	-10.281 -7.227	-11.542 -8.127	-10.947 -7.532	-10.387 -7.333	-4.354 -0.939	-5.423 -2.008
12	-4.877 -1.822	-6.617 -3.562	-7.878 -4.463	-7.282 -3.867	-6.723 -3.668	-0.689 2.725	-1.758 1.657
1 13	-7.552 -4.497	-9.292 -6.238	-10.553 -7.138	-9.958 -6.543	-9.398 -6.344	-3.365 0.050	-4.433 -1.019
14	-8.346 -5.292	-10.086 -7.032	-11.347 -7.932	-10.752 -7.337	-10.192 -7.138	-4.159 -0.744	-5.228 -1.813

	8	9	10	11	12	13
9	-0.198 3.217					
10	-1.478 1.937	-2.988 0.427				
11	-1.881 1.534	-3.390 0.024	-2.110 1.305			
12	1.784 5.199	0.274 3.689	1.555 4.970	1.957 5.372		
13	-0.891 2.523	-2.401 1.014	-1.120 2.294	-0.718 2.697	-4.383 -0.968	
14	-1.686 1.729	-3.195 0.219	-1.915 1.500	-1.512 1.902	-5.177 -1.762	-2.502 0.913

Fisher's pairwise comparisons

Family error rate = 0.738
Individual error rate = 0.0500

Critical value = 2.032

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5	6	7
2	0.995						
	2.485						
3	1.960	0.220					
	3.681	1.941					
4	1.365	-0.375	-1.558				
	3.086	1.346	0.367				
5	1.101	-0.639	-1.835	-1.240			
	2.591	0.851	-0.114	0.481			
1							
6	-5.228	-6.968	-8.150	-7.555	-7.074		
	-3.507	-5.247	-6.226	-5.631	-5.353		
7	-4.159	-5.899	-7.082	-6.486	-6.005	0.107	
	-2.438	-4.178	-5.157	-4.562	-4.284	2.031	
8	-7.701	-9.441	-10.624	-10.028	-9.547	-3.435	-4.504
	-5.980	-7.720	-8.700	-8.104	-7.826	-1.511	-2.580
9	-6.191	-7.932	-9.114	-8.518	-8.037	-1.926	-2.994
	-4.470	-6.211	-7.190	-6.594	-6.316	-0.001	-1.070
10	-7.472	-9.212	-10.395	-9.799	-9.318	-3.206	-4.275
	-5.751	-7.491	-8.471	-7.875	-7.597	-1.282	-2.351
11	-7.874	-9.615	-10.797	-10.201	-9.720	-3.609	-4.677
	-6.153	-7.894	-8.873	-8.277	-7.999	-1.684	-2.753
12	-4.210	-5.950	-7.132	-6.537	-6.056	0.056	-1.013
	-2.489	-4.229	-5.208	-4.613	-4.335	1.980	0.911
13	-6.885	-8.625	-9.808	-9.212	-8.731	-2.619	-3.688
	-5.164	-6.904	-7.884	-7.288	-7.010	-0.695	-1.764
14	-7.679	-9.420	-10.602	-10.006	-9.525	-3.414	-4.482
	-5.958	-7.699	-8.678	-8.082	-7.804	-1.489	-2.558

	8	9	10	11	12	13
9	0.548					
	2.472					
10	-0.733	-2.243				
	1.191	-0.319				
11	-1.135	-2.645	-1.364			
	0.789	-0.721	0.560			
12	2.529	1.019	2.300	2.702		
	4.453	2.944	4.224	4.627		
1						
13	-0.146	-1.656	-0.375	0.027	-3.637	
	1.778	0.268	1.549	1.951	-1.713	
14	-0.940	-2.450	-1.169	-0.767	-4.432	-1.756
	0.984	-0.526	0.755	1.157	-2.507	0.168

Appendix 1.2: Data of c-AMP in α-MSH analogues at B-16 Melanoma Cells.

ROW	C1	C2	C3
1	1	28	3.3322
2	1	32	3.4657
3	1	33	3.4965
4	1	20	2.9806
5	1	13	2.5572
6	1	39	3.6636
7	2	4	1.3913
8	2	1	-0.3147
9	2	1	-0.5604
10	2	1	0.3920
11	2	2	0.8020
12	2	1	-0.0010
13	3	2	0.5710

```
  14      3         6    1.8148
  15      3         7    1.9473
  16      3         9    2.1702
  17      3         6    1.7901
  18      4         3    1.1756
  19      4         4    1.4398
  20      4         2    0.5766
  21      5         4    1.4303
  22      5         3    0.9478
  23      5         1    0.2852
  24      5         1    0.3577
  25      6      1310    7.1778
  26      6       841    6.7346
  27      6      4620    8.4382
  28      7      4000    8.2940
  29      7      6300    8.7483
  30      7      3210    8.0740
  31      8    194000   12.1756
  32      8    576000   13.2639
  33      8    148000   11.9050
  34      9     59400   10.9920
  35      9     45700   10.7299
  36      9     28900   10.2716
  37     10     10000    9.2103
  38     11     40000   10.5966
  39     11     80000   11.2898
  40     12      2440    7.7998
  41     12      2200    7.6962
  42     12       390    5.9661
  43     12       415    6.0283
  44     13     17900    9.7926
  45     13     41000   10.6213
  46     13     30300   10.3189
  47     14     68800   11.1390
  48     14     30000   10.3090
  49     14     13200    9.4880

MTB > onew c3 c1;
SUBC> fisher 0.05;
SUBC> tukey.


ANALYSIS OF VARIANCE ON C3
SOURCE     DF        SS        MS         F        p
C1         13   894.776    68.829    166.65    0.000
ERROR      35    14.456     0.413
TOTAL      48   909.232

                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -+---------+---------+---------+-----
     1      6     3.249     0.409           (*)
     2      6     0.285     0.729  (-*)
     3      5     1.659     0.626      (*-)
     4      3     1.064     0.442    (-*-)
     5      4     0.755     0.539   (-*-)
     6      3     7.450     0.884                     (-*-)
     7      3     8.372     0.344                       (-*-)
     8      3    12.448     0.719                                 (-*-)
     9      3    10.665     0.365                           (-*-)
    10      1     9.210     0.000                        (--*--)
    11      2    10.943     0.490                             (-*--)
    12      4     6.873     1.012                     (*-)
    13      3    10.244     0.419                           (-*)
    14      3    10.312     0.825                           (-*-)
                                  -+---------+---------+---------+-----
POOLED STDEV =    0.643           0.0       4.0       8.0      12.0

1
Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.000980

Critical value = 5.09

Intervals for (column level mean) - (row level mean)
```

	1	2	3	4	5	6	7
2	1.629						
	4.300						
3	0.190	-2.774					
	2.991	0.027					
4	0.550	-2.415	-1.095				
	3.821	0.856	2.284				
5	1.001	-1.963	-0.648	-1.458			
	3.987	1.023	2.455	2.075			
6	-5.836	-8.801	-7.481	-8.275	-8.462		
	-2.565	-5.530	-4.102	-4.498	-4.928		
7	-6.758	-9.723	-8.403	-9.197	-9.384	-2.811	
	-3.487	-6.452	-5.024	-5.419	-5.850	0.967	
8	-10.834	-13.799	-12.479	-13.273	-13.460	-6.887	-5.965
	-7.563	-10.528	-9.100	-9.496	-9.926	-3.109	-2.187
9	-9.051	-12.015	-10.695	-11.489	-11.676	-5.103	-4.181
	-5.780	-8.744	-7.317	-7.712	-8.143	-1.326	-0.404
10	-8.459	-11.424	-10.086	-10.817	-11.041	-4.431	-3.509
	-3.463	-6.427	-5.018	-5.475	-5.869	0.911	1.833
11	-9.583	-12.547	-11.220	-11.991	-12.191	-5.605	-4.683
	-5.805	-8.770	-7.349	-7.768	-8.185	-1.381	-0.460
12	-5.116	-8.081	-6.766	-7.575	-7.753	-1.189	-0.267
	-2.130	-5.095	-3.662	-4.042	-4.482	2.344	3.266

1

	1	2	3	4	5	6	7
13	-8.631	-11.595	-10.275	-11.069	-11.256	-4.683	-3.761
	-5.359	-8.324	-6.896	-7.292	-7.722	-0.905	0.016
14	-8.698	-11.663	-10.343	-11.137	-11.323	-4.750	-3.828
	-5.427	-8.391	-6.964	-7.359	-7.790	-0.973	-0.051

	8	9	10	11	12	13
9	-0.105					
	3.672					
10	0.567	-1.217				
	5.909	4.125				
11	-0.607	-2.390	-4.566			
	3.616	1.833	1.100			
12	3.809	2.025	-0.248	2.067		
	7.342	5.559	4.924	6.074		
13	0.315	-1.468	-3.705	-1.413	-5.138	
	4.093	2.309	1.637	2.810	-1.605	
14	0.248	-1.536	-3.773	-1.480	-5.206	-1.956
	4.025	2.241	1.569	2.743	-1.673	1.821

Fisher's pairwise comparisons

Family error rate = 0.739
Individual error rate = 0.0500

Critical value = 2.030

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5	6	7
2	2.211						
	3.718						
3	0.801	-2.164					
	2.381	-0.584					

```
      4     1.263    -1.702    -0.358
            3.108     0.143     1.547

      5     1.652    -1.312     0.028    -0.688
            3.336     0.372     1.779     1.305

1
      6    -5.123    -8.088    -6.744    -7.451    -7.691
           -3.278    -6.243    -4.839    -5.321    -5.699

      7    -6.045    -9.010    -7.666    -8.373    -8.613    -1.987
           -4.200    -7.165    -5.761    -6.243    -6.620     0.143

      8   -10.121   -13.086   -11.742   -12.449   -12.689    -6.063    -5.141
           -8.276   -11.241    -9.837   -10.319   -10.696    -3.933    -3.011

      9    -8.338   -11.302    -9.959   -10.666   -10.906    -4.280    -3.358
           -6.493    -9.457    -8.053    -8.535    -8.913    -2.149    -1.227

     10    -7.370   -10.335    -8.981    -9.653    -9.914    -3.267    -2.345
           -4.552    -7.516    -6.123    -6.640    -6.996    -0.254     0.668

     11    -8.759   -11.724   -10.376   -11.070   -11.318    -4.684    -3.762
           -6.629    -9.593    -8.193    -8.688    -9.058    -2.302    -1.380

     12    -4.465    -7.430    -6.089    -6.805    -7.040    -0.419     0.503
           -2.781    -5.746    -4.339    -4.812    -5.195     1.574     2.496

     13    -7.917   -10.882    -9.538   -10.245   -10.485    -3.859    -2.937
           -6.072    -9.037    -7.633    -8.115    -8.493    -1.729    -0.807

     14    -7.985   -10.950    -9.606   -10.313   -10.553    -3.927    -3.005
           -6.140    -9.105    -7.701    -8.183    -8.560    -1.797    -0.875


8          9         10         11         12         13

      9     0.718
            2.849

     10     1.731    -0.052
            4.744     2.961

     11     0.314    -1.470    -3.331
            2.696     0.912    -0.135

     12     4.579     2.795     0.879     2.941
            6.572     4.788     3.796     5.200

1
     13     1.139    -0.645    -2.540    -0.492    -4.368
            3.269     1.485     0.473     1.890    -2.375

     14     1.071    -0.713    -2.608    -0.560    -4.436    -1.133
            3.201     1.418     0.405     1.822    -2.443     0.998
```

Appendix 1.3: Data of Binding Assay in α-MSH analogues at MC3-R.

ROW	C1	C2	C3
1	1	48.0	3.87120
2	1	47.5	3.86073
3	1	102.0	4.62497
4	2	4.1	1.41342
5	2	2.1	0.75142
6	2	2.1	0.72271
7	2	1.0	0.01980
8	2	1.5	0.41211
9	3	2.1	0.72755
10	3	1.0	0.00000
11	3	1.0	0.03922
12	3	0.9	-0.13582
13	3	1.1	0.10436
14	4	1.1	0.13103
15	4	0.9	-0.12897
16	4	0.6	-0.52594
17	4	0.5	-0.60331
18	4	0.9	-0.05235
19	5	3.0	1.09527

20	5	1.5	0.38526
21	5	1.6	0.45108
22	5	1.3	0.22314
23	5	1.1	0.12222
24	5	1.3	0.27003
25	6	4810.0	8.47845
26	6	14400.0	9.57498
27	6	6440.0	8.77028
28	7	702.0	6.55393
29	7	475.0	6.16331
30	7	681.0	6.52356
31	8	4530.0	8.41848
32	8	14000.0	9.54681
33	8	4270.0	8.35937
34	9	1910.0	7.55486
35	9	2000.0	7.60090
36	9	2720.0	7.90839
37	9	4110.0	8.32118
38	10	1860.0	7.52833
39	10	1470.0	7.29302
40	10	1730.0	7.45588
41	11	2490.0	7.82004
42	11	3880.0	8.26359
43	11	3520.0	8.16622
44	12	1320.0	7.18539
45	12	1140.0	7.03878
46	12	3380.0	8.12563
47	13	7030.0	8.85794
48	13	4980.0	8.51319
49	13	2110.0	7.65444
50	14	4650.0	8.44462
51	14	3170.0	8.06149
52	14	2400.0	7.78322

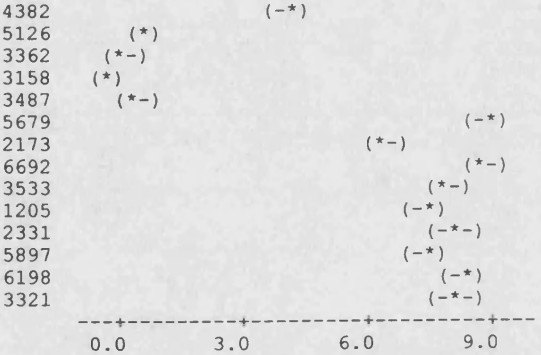
```
MTB > onew c3 c1;
SUBC> fisher 0.05;
SUBC> tukey.
```

.ANALYSIS OF VARIANCE ON C3

SOURCE	DF	SS	MS	F	p
C1	13	723.086	55.622	314.26	0.000
ERROR	38	6.726	0.177		
TOTAL	51	729.812			

LEVEL	N	MEAN	STDEV
1	3	4.1190	0.4382
2	5	0.6639	0.5126
3	5	0.1471	0.3362
4	5	-0.2359	0.3158
5	6	0.4245	0.3487
6	3	8.9412	0.5679
7	3	6.4136	0.2173
8	3	8.7749	0.6692
9	4	7.8463	0.3533
10	3	7.4257	0.1205
11	3	8.0833	0.2331
12	3	7.4499	0.5897
13	3	8.3419	0.6198
14	3	8.0964	0.3321

POOLED STDEV = 0.4207



Tukey's pairwise comparisons

Family error rate = 0.0500
Individual error rate = 0.000964
Critical value = 5.06

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5	6	7
2	2.3558 4.5544						
3	2.8726 5.0712	-0.4352 1.4689					
4	3.2556	-0.0522	-0.5691				

```
          5.4542    1.8518    1.3350

      5    2.6301   -0.6721   -1.1889   -1.5719
           4.7589    1.1509    0.6341    0.2511

      6   -6.0513   -9.3766   -9.8935  -10.2764   -9.5811
          -3.5932   -7.1780   -7.6949   -8.0778   -7.4523

      7   -3.5237   -6.8490   -7.3658   -7.7488   -7.0535    1.2986
          -1.0656   -4.6504   -5.1672   -5.5502   -4.9247    3.7567

      8   -5.8850   -9.2103   -9.7271  -10.1101   -9.4148   -1.0627   -3.5903
          -3.4269   -7.0117   -7.5285   -7.9115   -7.2860    1.3954   -1.1322

      9   -4.8770   -8.1922   -8.7090   -9.0920   -8.3935   -0.0548   -2.5824
          -2.5777   -6.1727   -6.6895   -7.0725   -6.4502    2.2446   -0.2831

     10   -4.5358   -7.8612   -8.3780   -8.7609   -8.0656    0.2864   -2.2412
          -2.0777   -5.6626   -6.1794   -6.5623   -5.9368    2.7446    0.2169

     11   -5.1934   -8.5187   -9.0355   -9.4185   -8.7232   -0.3711   -2.8987
          -2.7353   -6.3201   -6.8369   -7.2199   -6.5944    2.0870   -0.4406

     12   -4.5600   -7.8853   -8.4022   -8.7851   -8.0898    0.2623   -2.2654
          -2.1019   -5.6867   -6.2036   -6.5865   -5.9610    2.7204    0.1927

     13   -5.4519   -8.7773   -9.2941   -9.6771   -8.9817   -0.6297   -3.1573
          -2.9938   -6.5787   -7.0955   -7.4785   -6.8530    1.8284   -0.6992

     14   -5.2065   -8.5319   -9.0487   -9.4317   -8.7363   -0.3843   -2.9119
          -2.7484   -6.3333   -6.8501   -7.2331   -6.6076    2.0739   -0.4538


8         9        10        11        12        13

      9   -0.2211
           2.0782

     10    0.1201   -0.7291
           2.5782    1.5703

     11   -0.5375   -1.3866   -1.8866
           1.9207    0.9127    0.5715

     12    0.0959   -0.7533   -1.2532   -0.5957
           2.5540    1.5461    1.2049    1.8624

     13   -0.7960   -1.6452   -2.1452   -1.4876   -2.1210
           1.6621    0.6542    0.3129    0.9705    0.3371

     14   -0.5506   -1.3998   -1.8998   -1.2422   -1.8756   -0.9836
           1.9075    0.8996    0.5584    1.2159    0.5825    1.4745


 Fisher's pairwise comparisons

     Family error rate = 0.744
 Individual error rate = 0.0500

 Critical value = 2.024

 Intervals for (column level mean) - (row level mean)

                1         2         3         4         5         6         7

      2    2.8332
           4.0769

      3    3.3500   -0.0217
           4.5938    1.0554

      4    3.7330    0.3613   -0.1556
           4.9767    1.4383    0.9215

      5    3.0924   -0.2762   -0.7931   -1.1760
           4.2966    0.7550    0.2382   -0.1448

1
      6   -5.5175   -8.8992   -9.4160   -9.7990   -9.1189
```

	-4.1270	-7.6555	-8.1723	-8.5553	-7.9146		
7	-2.9899	-6.3716	-6.8884	-7.2714	-6.5912	1.8324	
	-1.5994	-5.1279	-5.6447	-6.0277	-5.3870	3.2229	
8	-5.3512	-8.7329	-9.2497	-9.6327	-8.9525	-0.5289	-3.0565
	-3.9607	-7.4891	-8.0060	-8.3889	-7.7483	0.8616	-1.6660
9	-4.3777	-7.7537	-8.2705	-8.6535	-7.9715	0.4446	-2.0831
	-3.0770	-6.6112	-7.1281	-7.5110	-6.8722	1.7453	-0.7824
10	-4.0020	-7.3837	-7.9005	-8.2835	-7.6034	0.8202	-1.7074
	-2.6115	-6.1400	-6.6568	-7.0398	-6.3991	2.2108	-0.3169
11	-4.6596	-8.0412	-8.5581	-8.9410	-8.2609	0.1627	-2.3649
	-3.2691	-6.7975	-7.3144	-7.6973	-7.0567	1.5532	-0.9744
12	-4.0262	-7.4079	-7.9247	-8.3077	-7.6275	0.7960	-1.7316
	-2.6357	-6.1642	-6.6810	-7.0640	-6.4233	2.1866	-0.3411
13	-4.9181	-8.2998	-8.8167	-9.1996	-8.5195	-0.0959	-2.6235
	-3.5276	-7.0561	-7.5729	-7.9559	-7.3152	1.2946	-1.2330
14	-4.6727	-8.0544	-8.5712	-8.9542	-8.2741	0.1495	-2.3781
	-3.2822	-6.8107	-7.3275	-7.7105	-7.0698	1.5401	-0.9876

8	9	10	11	12	13		
9	0.2782						
	1.5789						
10	0.6539	-0.2298					
	2.0444	1.0709					
11	-0.0037	-0.8873	-1.3528				
	1.3869	0.4134	0.0377				
12	0.6297	-0.2540	-0.7195	-0.0619			
	2.0202	1.0468	0.6711	1.3286			
13	-0.2622	-1.1459	-1.6114	-0.9538	-1.5872		
	1.1283	0.1548	-0.2209	0.4367	-0.1967		
14	-0.0168	-0.9005	-1.3660	-0.7084	-1.3418	-0.4498	
	1.3737	0.4002	0.0246	0.6821	0.0487	0.9407	

Appendix 1.4: Data of c-AMP in α-MSH analogues at MC3-R.

ROW	C1	C2	C3
1	1	1.990	0.68813
2	1	0.633	-0.45728
3	1	6.080	1.80500
4	1	0.748	-0.29035
5	2	0.043	-3.14888
6	2	0.126	-2.07147
7	2	0.207	-1.57504
8	3	0.506	-0.68122
9	3	0.433	-0.83702
10	3	0.136	-1.99510
11	4	0.283	-1.26231
12	4	0.426	-0.85332
13	4	0.110	-2.20727
14	5	0.473	-0.74866
15	5	0.183	-1.69827
16	5	0.212	-1.55117
17	6	5.130	1.63511
18	6	21.500	3.06805
19	6	3.330	1.20297
20	7	0.773	-0.25748
21	7	1.360	0.30748
22	7	0.670	-0.40048
23	8	2.410	0.87963
24	8	3.860	1.35067
25	8	56.500	4.03424
26	9	6.180	1.82132
27	9	4.530	1.51072
28	9	2.350	0.85442
29	10	9.560	2.25759

```
  30     10    42.200   3.74242
  31     10    68.200   4.22244
  32     10    56.900   4.04130
  33     11   136.000   4.91265
  34     11    64.800   4.17131
  35     11   136.000   4.91265
  36     12     8.740   2.16791
  37     12     1.560   0.44469
  38     12     3.000   1.09861
  39     13    20.700   3.03013
  40     13    20.700   3.03013
  41     13     5.150   1.63900
  42     14    82.200   4.40916
  43     14    92.300   4.52504
  44     14    14.000   2.63906

MTB > onew c3 c1;
SUBC> fisher 0.05;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C3
SOURCE     DF        SS        MS        F        p
C1         13   189.927    14.610    18.77    0.000
ERROR      30    23.346     0.778
TOTAL      43   213.272

                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ----+---------+---------+---------+--
     1      4    0.4364    1.0430              (---*--)
     2      3   -2.2651    0.8046   (---*---)
     3      3   -1.1711    0.7178       (---*---)
     4      3   -1.4410    0.6944      (---*---)
     5      3   -1.3327    0.5111       (---*---)
     6      3    1.9687    0.9763                  (---*---)
     7      3   -0.1168    0.3744           (----*---)
     8      3    2.0882    1.7017                  (---*----)
     9      3    1.3955    0.4936                (----*---)
    10      4    3.5659    0.8944                        (--*---)
    11      3    4.6655    0.4280                            (---*---)
    12      3    1.2371    0.8699                (---*---)
    13      3    2.5664    0.8032                     (---*---)
    14      3    3.8578    1.0570                           (---*----)
                                  ----+---------+---------+---------+--
POOLED STDEV =    0.8821           -2.5       0.0       2.5       5.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.00102
Critical value = 5.15

Intervals for (column level mean) - (row level mean)

              1         2         3         4         5         6         7

     2    0.2480
          5.1550

     3   -0.8460   -3.7170
          4.0610    1.5289

     4   -0.5762   -3.4471   -2.3531
          4.3309    1.7988    2.8928

     5   -0.6845   -3.5554   -2.4614   -2.7312
          4.2226    1.6905    2.7845    2.5147

     6   -3.9859   -6.8568   -5.7628   -6.0326   -5.9244
          0.9212   -1.6109   -0.5169   -0.7867   -0.6785

     7   -1.9003   -4.7712   -3.6772   -3.9471   -3.8388   -0.5374
          3.0067    0.4746    1.5687    1.2988    1.4071    4.7085

     8   -4.1053   -6.9763   -5.8822   -6.1521   -6.0438   -2.7424   -4.8279
          0.8017   -1.7304   -0.6363   -0.9062   -0.7979    2.5035    0.4179

     9   -3.4126   -6.2836   -5.1895   -5.4594   -5.3511   -2.0497   -4.1352
          1.4944   -1.0377    0.0563   -0.2135   -0.1052    3.1962    1.1106

    10   -5.4011   -8.2846   -7.1906   -7.4604   -7.3522   -4.0508   -6.1363
         -0.8580   -3.3775   -2.2835   -2.5534   -2.4451    0.8563   -1.2292

    11   -6.6827   -9.5536   -8.4596   -8.7294   -8.6212   -5.3198   -7.4053
```

	-1.7756	-4.3077	-3.2137	-3.4836	-3.3753	-0.0739	-2.1594
12	-3.2542	-6.1251	-5.0311	-5.3010	-5.1927	-1.8913	-3.9768
	1.6528	-0.8793	0.2148	-0.0551	0.0532	3.3546	1.2690
13	-4.5836	-7.4545	-6.3605	-6.6303	-6.5221	-3.2207	-5.3062
	0.3235	-2.2086	-1.1146	-1.3844	-1.2762	2.0252	-0.0603
14	-5.8749	-8.7458	-7.6518	-7.9217	-7.8134	-4.5120	-6.5975
	-0.9678	-3.4999	-2.4059	-2.6758	-2.5675	0.7339	-1.3516

	8	9	10	11	12	13
9	-1.9302					
	3.3156					
10	-3.9313	-4.6240				
	0.9758	0.2831				
11	-5.2003	-5.8930	-3.5531			
	0.0456	-0.6471	1.3539			
12	-1.7718	-2.4645	-0.1247	0.8055		
	3.4741	2.7814	4.7824	6.0514		

1

13	-3.1012	-3.7939	-1.4540	-0.5238	-3.9523	
	2.1447	1.4520	3.4531	4.7221	1.2936	
14	-4.3925	-5.0852	-2.7454	-1.8152	-5.2436	-3.9143
	0.8534	0.1607	2.1617	3.4307	0.0023	1.3316

Fisher's pairwise comparisons

Family error rate = 0.731
Individual error rate = 0.0500

Critical value = 2.042

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5	6	7
2	1.3257						
	4.0773						
3	0.2317	-2.5648					
	2.9833	0.3768					
4	0.5015	-2.2950	-1.2009				
	3.2531	0.6466	1.7406				
5	0.3933	-2.4032	-1.3092	-1.5791			
	3.1449	0.5384	1.6324	1.3625			
6	-2.9081	-5.7046	-4.6106	-4.8805	-4.7722		
	-0.1565	-2.7630	-1.6690	-1.9389	-1.8306		
7	-0.8226	-3.6191	-2.5251	-2.7949	-2.6867	0.6147	
	1.9290	-0.6775	0.4165	0.1467	0.2549	3.5563	
8	-3.0276	-5.8241	-4.7301	-4.9999	-4.8917	-1.5903	-3.6758
	-0.2760	-2.8825	-1.7885	-2.0583	-1.9501	1.3513	-0.7342
9	-2.3349	-5.1314	-4.0374	-4.3072	-4.1990	-0.8976	-2.9831
	0.4167	-2.1898	-1.0958	-1.3657	-1.2574	2.0440	-0.0415
10	-4.4033	-7.2069	-6.1129	-6.3827	-6.2744	-2.9730	-5.0586
	-1.8558	-4.4553	-3.3612	-3.6311	-3.5228	-0.2214	-2.3070
11	-5.6050	-8.4015	-7.3074	-7.5773	-7.4690	-4.1676	-6.2532
	-2.8534	-5.4599	-4.3659	-4.6357	-4.5274	-1.2260	-3.3116
12	-2.1765	-4.9730	-3.8790	-4.1488	-4.0406	-0.7392	-2.8247
	0.5751	-2.0314	-0.9374	-1.2072	-1.0990	2.2024	0.1169

13	-3.5058	-6.3023	-5.2083	-5.4782	-5.3699	-2.0685	-4.1540
	-0.7542	-3.3608	-2.2667	-2.5366	-2.4283	0.8731	-1.2124
14	-4.7972	-7.5937	-6.4997	-6.7695	-6.6612	-3.3598	-5.4454
	-2.0456	-4.6521	-3.5581	-3.8279	-3.7197	-0.4182	-2.5038

	8	9	10	11	12	13
9	-0.7781					
	2.1635					
10	-2.8536	-3.5463				
	-0.1020	-0.7946				
11	-4.0482	-4.7408	-2.4754			
	-1.1066	-1.7993	0.2762			
12	-0.6197	-1.3124	0.9531	1.9577		
	2.3219	1.6292	3.7047	4.8993		
13	-1.9490	-2.6417	-0.3763	0.6283	-2.8001	
	0.9926	0.2999	2.3753	3.5699	0.1414	
14	-3.2404	-3.9331	-1.6676	-0.6630	-4.0915	-2.7621
	-0.2988	-0.9915	1.0840	2.2786	-1.1499	0.1795

Appendix 1.5: Data of binding Assay for Multiple Alanine Substitution Analogues in α-MSH at B-16 Melanoma Cells.

ROW	C1	C2	C3
1	1	1.040	0.03922
2	1	1.890	0.63658
3	1	1.620	0.48243
4	1	1.860	0.62058
5	1	2.010	0.69813
6	2	0.550	-0.59784
7	2	0.142	-1.95193
8	2	0.480	-0.73397
9	2	0.247	-1.39837
10	2	0.214	-1.54178
11	3	35.000	3.55535
12	3	43.500	3.77276
13	3	27.500	3.31419
14	4	3.820	1.34025
15	4	4.830	1.57485
16	4	1.670	0.51282
17	5	6.640	1.89311
18	5	4.130	1.41828
19	5	2.210	0.79299
20	6	1.560	0.44469
21	6	1.280	0.24686
22	6	8.730	2.16677

```
MTB >
MTB > onew c3 c1;
SUBC> fisher 0.05;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C3
SOURCE     DF        SS        MS        F        p
C1          5    45.401     9.080    28.25    0.000
ERROR      16     5.143     0.321
TOTAL      21    50.544

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ---------+---------+---------+-------
     1      5    0.4954    0.2669            (-*--)
     2      5   -1.2448    0.5682  (--*-)
     3      3    3.5474    0.2294                              (---*--)
     4      3    1.1426    0.5579               (---*--)
     5      3    1.3681    0.5518                (---*--)
     6      3    0.9528    1.0560             (---*--)
                                    ---------+---------+---------+-------
 POOLED STDEV =    0.5670                  0.0       2.0       4.0

1
Tukey's pairwise comparisons
     Family error rate = 0.0500
 Individual error rate = 0.00530
```

Critical value = 4.5

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5
2	0.5840 2.8964				
3	-4.3871 -1.7170	-6.1273 -3.4571			
4	-1.9823 0.6878	-3.7225 -1.0523	0.9121 3.8975		
5	-2.2078 0.4623	-3.9480 -1.2778	0.6866 3.6720	-1.7181 1.2672	
6	-1.7925 0.8777	-3.5326 -0.8625	1.1020 4.0873	-1.3028 1.6825	-1.0773 1.9080

Fisher's pairwise comparisons

Family error rate = 0.326
Individual error rate = 0.0500

Critical value = 2.120

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5
2	0.9800 2.5004				
3	-3.9298 -2.1743	-5.6700 -3.9144			
4	-1.5250 0.2305	-3.2652 -1.5096	1.4234 3.3862		
5	-1.7505 0.0051	-3.4907 -1.7351	1.1979 3.1607	-1.2069 0.7559	
1 6	-1.3352 0.4204	-3.0753 -1.3198	1.6133 3.5761	-0.7915 1.1713	-0.5660 1.3968

Appendix 1.6: Data of c-AMP for Multiple Alanine Substitution Analogues in α-MSH at B-16 Melanoma Cells.

ROW	C1	C2	C3
1	1	28.00	3.33220
2	1	32.00	3.46574
3	1	33.00	3.49651
4	1	19.70	2.98062
5	1	12.90	2.55723
6	1	39.00	3.66356
7	2	4.02	1.39128
8	2	0.73	-0.31471
9	2	0.57	-0.56037
10	2	1.48	0.39204
11	2	2.23	0.80200
12	2	1.00	-0.00100
13	3	2230.00	7.70976
14	3	6880.00	8.83637
15	3	2250.00	7.71869
16	4	423.00	6.04737
17	4	342.00	5.83481
18	4	131.00	4.87520
19	5	593.00	6.38519
20	5	749.00	6.61874
21	5	837.00	6.72982
22	6	1560.00	7.35244
23	6	4500.00	8.41183
24	6	5150.00	8.54675

```
MTB > onew c3 c1;
SUBC> fisher 0.05;
SUBC> tud
SUBC> tukey.

ANALYSIS OF VARIANCE ON C3
SOURCE     DF        SS        MS        F        p
C1          5   209.941    41.988   125.27    0.000
ERROR      18     6.033     0.335
TOTAL      23   215.975

                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV -+---------+---------+---------+-----
     1      6    3.2493    0.4093              (-*-)
     2      6    0.2849    0.7292  (-*-)
     3      3    8.0883    0.6479                                   (-*--)
     4      3    5.5858    0.6245                         (-*--)
     5      3    6.5779    0.1759                             (-*--)
     6      3    8.1037    0.6541                                   (-*--)
                                  -+---------+---------+---------+-----
POOLED STDEV =    0.5790          0.0       2.5       5.0       7.5

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.00524

Critical value = 4.49

Intervals for (column level mean) - (row level mean)

               1         2         3         4         5

     2    1.9032
          4.0257

     3   -6.1387   -9.1031
         -3.5392   -6.5036

     4   -3.6362   -6.6007    1.0017
         -1.0367   -4.0012    4.0033

     5   -4.6284   -7.5928    0.0095   -2.4929
         -2.0289   -4.9933    3.0112    0.5087

1
     6   -6.1541   -9.1185   -1.5162   -4.0187   -3.0266
         -3.5546   -6.5191    1.4854   -1.0171   -0.0249


Fisher's pairwise comparisons

    Family error rate = 0.330
Individual error rate = 0.0500

Critical value = 2.101

Intervals for (column level mean) - (row level mean)

               1         2         3         4         5

     2    2.2622
          3.6667

     3   -5.6991   -8.6635
         -3.9789   -6.9433

     4   -3.1966   -6.1610    1.5093
         -1.4764   -4.4408    3.4956

     5   -4.1887   -7.1532    0.5172   -1.9853
         -2.4685   -5.4329    2.5035    0.0010

     6   -5.7145   -8.6789   -1.0086   -3.5111   -2.5189
         -3.9943   -6.9587    0.9778   -1.5247   -0.5326
```

Appendix 1.7: Data of Binding Assay for Alanine Substitution Analogues in α-MSH at MC3-R.

ROW	C1	C2	C3
1	1	83.90	4.42963
2	1	68.30	4.22391
3	1	74.50	4.31080
4	2	4.11	1.41342
5	2	2.12	0.75142
6	2	2.06	0.72271
7	2	1.02	0.01980
8	2	1.51	0.41211
9	3	1770.00	7.47873
10	3	1930.00	7.56528
11	3	2070.00	7.63530
12	3	2420.00	7.79152
13	3	1550.00	7.34601
14	4	944.00	6.85013
15	4	728.00	6.59030
16	4	285.00	5.65249
17	4	836.00	6.72863
18	4	843.00	6.73697
19	5	107.00	4.67283
20	5	125.00	4.82831
21	5	84.90	4.44147
22	6	1280.00	7.15462
23	6	1950.00	7.57558
24	6	1020.00	6.92756

```
MTB > onew c3 c1;
SUBC> fisher 0.05;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C3
SOURCE     DF        SS        MS        F        p
C1          5   154.606    30.921   228.77    0.000
ERROR      18     2.433     0.135
TOTAL      23   157.039

                                INDIVIDUAL 95 PCT CI'S FOR MEAN
                                BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ---------+---------+---------+-------
     1      3    4.3214    0.1033                (*-)
     2      5    0.6639    0.5126  (-*)
     3      5    7.5634    0.1671                                  (*-)
     4      5    6.5117    0.4891                            (*)
     5      3    4.6475    0.1947                   (-*)
     6      3    7.2193    0.3288                                (-*-)
                                ---------+---------+---------+-------
POOLED STDEV =    0.3676                2.5       5.0       7.5

1
Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.00524

Critical value = 4.49

Intervals for (column level mean) - (row level mean)

               1         2         3         4         5

     2    2.8051
          4.5100

     3   -4.0944   -7.6377
         -2.3895   -6.1613

     4   -3.0427   -6.5860    0.3134
         -1.3378   -5.1096    1.7899

     5   -1.2791   -4.8361    2.0634    1.0117
          0.6269   -3.1312    3.7683    2.7166

     6   -3.8509   -7.4078   -0.5083   -1.5600   -3.5248
         -1.9448   -5.7029    1.1965    0.1449   -1.6187


Fisher's pairwise comparisons
```

```
     Family error rate = 0.330
 Individual error rate = 0.0500

 Critical value = 2.101

 Intervals for (column level mean) - (row level mean)

                1         2         3         4         5

      2    3.0935
           4.2216

      3   -3.8060   -7.3880
          -2.6778   -6.4110

      4   -2.7544   -6.3363    0.5631
          -1.6262   -5.3593    1.5402

      5   -0.9568   -4.5477    2.3517    1.3001
           0.3046   -3.4196    3.4799    2.4283

1
      6   -3.5285   -7.1195   -0.2200   -1.2716   -3.2024
          -2.2671   -5.9913    0.9082   -0.1435   -1.9410
```

Appendix 1.8: Data of c-AMP for Alanine Substitution Analogues in α-MSH at MC3-R.

```
 ROW   C1       C2        C3

   1    1   0.2590  -1.35093
   2    1   0.5220  -0.65009
   3    1   6.6600   1.89612
   4    2   0.0429  -3.14888
   5    2   0.1260  -2.07147
   6    2   0.2070  -1.57504
   7    3   2.5400   0.93216
   8    3   2.7500   1.01160
   9    3   3.3200   1.19996
  10    3   4.2500   1.44692
  11    4   4.6500   1.53687
  12    4   2.8700   1.05431
  13    4   2.6700   0.98208
  14    4   1.9900   0.68813
  15    4   1.9400   0.66269
  16    5   0.3830  -0.95972
  17    5   0.3360  -1.09064
  18    5   0.9670  -0.03356
  19    6   1.1200   0.11333
  20    6   1.7200   0.54232
  21    6   1.8800   0.63127

MTB > onew c3 c1;
SUBC> fisher 0.05;
 SUBC> tukey.

 ANALYSIS OF VARIANCE ON C3
 SOURCE     DF        SS        MS        F        p
 C1          5    27.356     5.471     9.53    0.000
 ERROR      15     8.610     0.574
 TOTAL      20    35.966

                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
  LEVEL      N      MEAN     STDEV  --+---------+---------+---------+----
      1      3   -0.0350    1.7087                  (-----*-----)
      2      3   -2.2651    0.8046   (-----*-----)
      3      4    1.1477    0.2289                            (-----*-----)
      4      5    0.9848    0.3540                            (----*---)
      5      3   -0.6946    0.5762             (-----*------)
      6      3    0.4290    0.2770                       (-----*-----)
                                  --+---------+---------+---------+----
 POOLED STDEV =   0.7576           -3.0       -1.5        0.0        1.5

 Tukey's pairwise comparisons

     Family error rate = 0.0500
 Individual error rate = 0.00543

 Critical value = 4.59

 Intervals for (column level mean) - (row level mean)
```

```
                1         2         3         4         5

     2     0.2224
           4.2379

     3    -3.0607   -5.2909
           0.6955   -1.5347

     4    -2.8156   -5.0457   -1.4867
           0.7760   -1.4542    1.8124

     5    -1.3481   -3.5782   -0.0358   -0.1163
           2.6674    0.4373    3.7204    3.4752

1
     6    -2.4717   -4.7019   -1.1594   -1.2400   -3.1314
           1.5438   -0.6863    2.5968    2.3516    0.8841


 Fisher's pairwise comparisons

     Family error rate = 0.323
 Individual error rate = 0.0500

 Critical value = 2.131

 Intervals for (column level mean) - (row level mean)

                1         2         3         4         5

     2     0.9119
           3.5484

     3    -2.4157   -4.6459
           0.0505   -2.1797

     4    -2.1989   -4.4290   -0.9202
           0.1593   -2.0709    1.2459

     5    -0.6586   -2.8887    0.6092    0.5004
           1.9779   -0.2522    3.0754    2.8585

     6    -1.7822   -4.0124   -0.5144   -0.6232   -2.4419
           0.8543   -1.3759    1.9518    1.7349    0.1946
1
```

Appendix 1.9: Data of Binding Assay for [L/D-Tyr^7]α-MSH, [L/D-Trp^7]α-MSH in at B-16 Melanoma Cells.

ROW	C1	C2	C3
1	1	10.40	2.34181
2	1	18.90	2.93916
3	1	16.20	2.78501
4	1	18.60	2.92316
5	1	20.10	3.00072
6	2	5.50	1.70475
7	2	1.42	0.35066
8	2	4.80	1.56862
9	2	2.47	0.90422
10	2	2.14	0.76081
11	3	117.00	4.76217
12	3	209.00	5.34233
13	3	245.00	5.50126
14	4	7850.00	8.96827
15	4	6040.00	8.70616
16	4	3150.00	8.05516
17	5	3.78	1.32972
18	5	5.92	1.77834
19	5	5.53	1.71019
20	5	3.16	1.15057
21	5	5.21	1.65058
22	6	8.83	2.17815
23	6	21.60	3.07269
24	6	20.70	3.03013
25	6	10.20	2.32239

```
 MTB > onew c3 c1;
 SUBC> fisher 0.05;
 SUBC> tukey.
```

```
ANALYSIS OF VARIANCE ON C3
SOURCE     DF        SS        MS        F        p
C1          5   137.696    27.539   160.17    0.000
ERROR      19     3.267     0.172
TOTAL      24   140.963

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  --------+---------+---------+--------
     1      5    2.7980    0.2669          (*-)
     2      5    1.0578    0.5682   (*-)
     3      3    5.2019    0.3890                    (-*-)
     4      3    8.5765    0.4702                                (-*-)
     5      5    1.5239    0.2704     (*-)
     6      4    2.6508    0.4666         (-*)
                                    --------+---------+---------+--------
POOLED STDEV =    0.4147                 2.5       5.0       7.5


Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.00515

Critical value = 4.47

Intervals for (column level mean) - (row level mean)

               1         2         3         4         5

     2    0.9113
          2.5691

     3   -3.3611   -5.1013
         -1.4468   -3.1870

     4   -6.7357   -8.4759   -4.4447
         -4.8214   -6.5616   -2.3045

     5    0.4452   -1.2950    2.7209    6.0955
          2.1030    0.3628    4.6352    8.0098

     6   -0.7321   -2.4722    1.5501    4.9247   -2.0062
          1.0263   -0.7138    3.5521    6.9267   -0.2478

Fisher's pairwise comparisons

    Family error rate = 0.332
Individual error rate = 0.0500

Critical value = 2.093

Intervals for (column level mean) - (row level mean)

               1         2         3         4         5

     2    1.1913
          2.2890

     3   -3.0377   -4.7779
         -1.7702   -3.5103

     4   -6.4124   -8.1525   -4.0832
         -5.1448   -6.8849   -2.6660

     5    0.7252   -1.0150    3.0442    6.4188
          1.8230    0.0828    4.3118    7.6864

     6   -0.4351   -2.1752    1.8882    5.2628   -1.7091
          0.7293   -1.0109    3.2139    6.5885   -0.5448
```

Appendix 1.10: Data of c-AMP for [L/D-Tyr^7]α-MSH,[L/D-Trp^7] α-MSH at B-16 Melanoma Cells.

ROW	C1	C2	C
1	1	2.80	1.02962
2	1	3.20	1.16315
3	1	3.30	1.19392
4	1	1.97	0.67803
5	1	1.29	0.25464
6	1	3.90	1.36098
7	2	0.40	-0.91130

```
   8     2       0.07  -2.61730
   9     2       0.06  -2.86295
  10     2       0.15  -1.91054
  11     2       0.22  -1.50058
  12     2       0.10  -2.30359
  13     3    4870.00   8.49085
  14     3    1960.00   7.58070
  15     3    4310.00   8.36869
  16     3    9390.00   9.14740
  17     4    2150.00   7.67322
  18     4    2230.00   7.70976
  19     4    2310.00   7.74500
  20     4    1350.00   7.20786
  21     5       9.22   2.22137
  22     5       1.98   0.68310
  23     5       3.52   1.25846
  24     6      14.80   2.69463
  25     6      22.80   3.12676
  26     6      11.90   2.47654

MTB > onew c3 c1;
SUBC> fisher 0.05
* NOTE * Subcommand does not end in . or ; (; assumed).
SUBC> fisher 0.05;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C3
SOURCE     DF        SS        MS        F        p
C1          5   382.394    76.479   240.74    0.000
ERROR      20     6.354     0.318
TOTAL      25   388.747

                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  --------+---------+---------+--------
     1      6    0.9467    0.4093           (-*)
     2      6   -2.0177    0.7292   (*-)
     3      4    8.3969    0.6427                                    (-*-)
     4      4    7.5840    0.2524                                  (-*)
     5      3    1.3876    0.7772            (-*-)
     6      3    2.7660    0.3309                (-*-)
                                  --------+---------+---------+--------
POOLED STDEV =   0.5636               0.0       3.5       7.0


Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.00508

Critical value = 4.45
 Intervals for (column level mean) - (row level mean)

               1         2         3         4         5

     2     1.940
           3.988

     3    -8.595   -11.559
          -6.305    -9.270

     4    -7.782   -10.746    -0.441
          -5.492    -8.457     2.067

     5    -1.695    -4.659     5.655     4.842
           0.813    -2.151     8.364     7.551

     6    -3.073    -6.038     4.276     3.463    -2.826
          -0.565    -3.530     6.986     6.173     0.070


Fisher's pairwise comparisons

    Family error rate = 0.333
Individual error rate = 0.0500

Critical value = 2.086

Intervals for (column level mean) - (row level mean)
```

	1	2	3	4	5
2	2.286				
	3.643				
3	-8.209	-11.174			
	-6.691	-9.656			
4	-7.396	-10.361	-0.018		
	-5.878	-8.843	1.644		
5	-1.272	-4.237	6.111	5.298	
	0.390	-2.574	7.907	7.094	
1					
6	-2.651	-5.615	4.733	3.920	-2.338
	-0.988	-3.952	6.529	5.716	-0.418

Appendix 1.11: Data of Binding Assay for [L/D-Tyr^7]α-MSH,[L/D-Trp^7]α-MSH at MC3-R.

ROW	C1	C2	C3
1	1	4.8000	1.56862
2	1	4.7500	1.55814
3	1	10.2000	2.32239
4	2	0.4110	-0.88916
5	2	0.2120	-1.55117
6	2	0.2060	-1.57988
7	2	0.1020	-2.28278
8	2	0.1510	-1.89048
9	3	0.0452	-3.09666
10	3	0.0428	-3.15122
11	3	0.0537	-2.92434
12	3	0.0515	-2.96617
13	4	0.0133	-4.31999
14	4	0.0285	-3.55785
15	4	0.0172	-4.06110
16	4	0.0245	-3.70908
17	5	2.9700	1.08856
18	5	3.8400	1.34547
20	5	5.8500	1.76644
21	6	2.7800	1.02245
22	6	2.8500	1.04732
23	6	4.2800	1.45395

```
MTB > onew c3 c1;
SUBC> fisher 0.05;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C3
SOURCE     DF        SS        MS        F        p
C1          5   115.424    23.085   170.00    0.000
ERROR      17     2.308     0.136
TOTAL      22   117.733

                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  --+---------+---------+---------+----
     1      3    1.8164    0.4382                                  (-*-)
     2      5   -1.6387    0.5126                 (-*-)
     3      4   -3.0346    0.1069          (-*-)
     4      4   -3.9120    0.3441  (-*-)
     5      4    1.5050    0.3494                                (-*)
     6      3    1.1746    0.2423                               (-*-)
                                  --+---------+---------+---------+----
POOLED STDEV =   0.3685          -4.0       -2.0       0.0       2.0


Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.00529

Critical value = 4.52
```

```
Intervals for (column level mean) - (row level mean)

               1         2         3         4         5

     2    2.5949
          4.3152

     3    3.9514    0.6058
          5.7505    2.1860

     4    4.8288    1.4832    0.0446
          6.6279    3.0634    1.7102

     5   -0.5882   -3.9338   -5.3725   -6.2499
          1.2109   -2.3537   -3.7068   -4.5842

1
     6   -0.3198   -3.6734   -5.1087   -5.9861   -0.5691
          1.6035   -1.9531   -3.3096   -4.1870    1.2300


Fisher's pairwise comparisons

    Family error rate = 0.328
Individual error rate = 0.0500

Critical value = 2.110

Intervals for (column level mean) - (row level mean)

               1         2         3         4         5

     2    2.8872
          4.0229

     3    4.2571    0.8743
          5.4448    1.9175

     4    5.1345    1.7517    0.3276
          6.3222    2.7949    1.4272

     5   -0.2825   -3.6653   -5.0894   -5.9669
          0.9052   -2.6221   -3.9898   -4.8672

     6    0.0069   -3.3811   -4.8030   -5.6804   -0.2634
          1.2767 -2.2454   -3.6153   -4.4927    0.9243
```

Appendix 1.12: Data of c-AMP for [L/D-Tyr^7]α-MSH at MC-3R.

ROW	C1	C2	C3
1	1	1.990	0.68813
2	1	0.633	-0.45728
3	1	6.080	1.80500
4	1	0.748	-0.29035
5	2	0.043	-3.14888
6	2	0.126	-2.07147
7	2	0.207	-1.57504
8	3	254.000	5.53733
9	3	75.900	4.32942
10	3	142.000	4.95583
11	3	41.800	3.73290
12	4	2.700	0.99325
13	4	1.750	0.55962
14	4	3.530	1.26130

```
MTB > onew c3 c1;
SUBC> fisher 0.05;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C3
SOURCE     DF        SS        MS        F        p
C1          3    85.847    28.616    43.14    0.000
ERROR      10     6.633     0.663
TOTAL      13    92.480

                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
```

```
 LEVEL      N      MEAN     STDEV  ----+---------+---------+---------+--
     1      4    0.4364    1.0430             (---*--)
     2      3   -2.2651    0.8046   (---*---)
     3      4    4.6389    0.7798                                (---*--)
     4      3    0.9381    0.3541                  (---*---)
                                   ----+---------+---------+---------+--
POOLED STDEV =   0.8144             -2.5       0.0       2.5       5.0

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0120

Critical value = 4.33

Intervals for (column level mean) - (row level mean)

               1         2         3

     2    0.7970
          4.6060

     3   -5.9657   -8.8085
         -2.4392   -4.9995

     4   -2.4062   -5.2392    1.7963
          1.4028   -1.1672    5.6053

Fisher's pairwise comparisons

    Family error rate = 0.181
Individual error rate = 0.0500

Critical value = 2.228


Intervals for (column level mean) - (row level mean)

               1         2         3

     2    1.3156
          4.0874

     3   -5.4856   -8.2899
         -2.9194   -5.5181

     4   -1.8876   -4.6848    2.3149
          0.8842   -1.7216    5.0867
```

Appendix 1.13: Data of c-AMP Antagonist for [L/D-Trp^7]α-MSH at MC3-R.

```
 ROW   C1       C2        C3

   1    1     6.12   1.81156
   2    1     3.67   1.30019
   3    1     7.21   1.97547
   4    2   124.00   4.82028
   5    2   244.00   5.49717
   6    2   218.00   5.38450

MTB > onew c3 c1;
SUBC> fisher 0.05;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C3
SOURCE     DF        SS        MS        F        p
C1          1    18.779    18.779   146.94    0.000
ERROR       4     0.511     0.128
TOTAL       5    19.290

                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ---+---------+---------+---------+---
     1      3    1.6957    0.3522  (---*---)
     2      3    5.2340    0.3627                        (---*---)
                                   ---+---------+---------+---------+---
POOLED STDEV =   0.3575             1.5       3.0       4.5       6.0

Tukey's pairwise comparisons
```

```
    Family error rate = 0.0500
Individual error rate = 0.0500

Critical value = 3.93

Intervals for (column level mean) - (row level mean)

               1

     2   -4.3494
         -2.7271
1
Fisher's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0500

Critical value = 2.776

Intervals for (column level mean) - (row level mean)

               1

     2   -4.3485
         -2.7280
```

Appendix 1.14: Data of Binding Assay for [L/D-Trp^7]α-MSH at MC4-R.

ROW	C1	C2	C3
1	1	108.00	4.68213
2	1	114.00	4.73620
3	1	192.00	5.25750
4	2	6.50	1.87180
5	2	4.51	1.50630
6	2	4.22	1.43984
7	3	457.00	6.12468
8	3	359.00	5.88332
9	4	49.90	3.91002
10	4	42.20	3.74242
11	4	43.80	3.779

```
MTB > onew c3 c1;
SUBC> fisher 0.05;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C3
SOURCE     DF        SS        MS        F        p
C1          3   27.6169    9.2056   181.66    0.000
ERROR       7    0.3547    0.0507
TOTAL      10   27.9717

                                 INDIVIDUAL 95 PCT CI'S FOR MEAN
                                 BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  --+---------+---------+---------+----
     1      3    4.8919    0.3177                        (-*-)
     2      3    1.6060    0.2326   (-*-)
     3      2    6.0040    0.1707                                 (-*--)
     4      3    3.8107    0.0880              (-*-)
                                 --+---------+---------+---------+----
POOLED STDEV =   0.2251           1.5       3.0       4.5       6.0

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0130

Critical value = 4.68

Intervals for (column level mean) - (row level mean)

               1         2         3

     2    2.6777
          3.8942

     3   -1.7921   -5.0781
         -0.4320   -3.7180

     4    0.4730   -2.8130    1.5133
          1.6895   -1.5965    2.8734
```

```
 Fisher's pairwise comparisons

     Family error rate = 0.172
 Individual error rate = 0.0500

 Critical value = 2.366

Intervals for (column level mean) - (row level mean)

                1         2         3

      2     2.8511
            3.7208

      3    -1.5983   -4.8842
           -0.6259   -3.9118

      4     0.6464   -2.6396    1.7071
            1.5161 -1.7698     2.6795
```

Appendix 1.15: Data of c-AMP for [L/D-Trp^7]α-MSH at MC4-R.

```
  ROW   C1       C2        C3

    1    1    0.752  -0.28502
    2    1    0.511  -0.67139
    3    1    1.150   0.13976
    4    2   17.300   2.85071
    5    2   12.500   2.52573
    6    2   15.700   2.75366
    7    3    5.260   1.66013
    8    3    2.880   1.05779
    9    3    7.550   2.02155

 MTB > onew c3 c1;
 SUBC> fisher 0.05;
 SUBC> tukey.

 ANALYSIS OF VARIANCE ON C3
 SOURCE     DF        SS        MS         F        p
 C1          2    13.601     6.801     47.50    0.000
 ERROR       6     0.859     0.143
 TOTAL       8    14.460

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
  LEVEL      N      MEAN     STDEV  -------+---------+---------+---------
      1      3   -0.2722    0.4057  (----*---)
      2      3    2.7100    0.1668                          (----*---)
      3      3    1.5798    0.4869                   (---*----)
                                    -------+---------+---------+---------
 POOLED STDEV =   0.3784                 0.0       1.2       2.4

 Tukey's pairwise comparisons

     Family error rate = 0.0500
 Individual error rate = 0.0220

 Critical value = 4.34
Intervals for (column level mean) - (row level mean)

                1         2

      2    -3.9303
           -2.0342

      3    -2.8001    0.1821
           -0.9040    2.0783


Fisher's pairwise comparisons

     Family error rate = 0.109
 Individual error rate = 0.0500

 Critical value = 2.448
 Intervals for (column level mean) - (row level mean)
```

```
              1         2

     2   -3.7385
         -2.2260

     3   -2.6083    0.3739
         -1.0958    1.8865
```

Appendix 1.16: Data of Binding Assay for Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-13}$ at B-16 Cells.

```
 ROW   C1       C2        C3

   1    1   10.400   2.34181
   2    1   18.900   2.93916
   3    1   16.200   2.78501
   4    1   18.600   2.92316
   5    1   20.100   3.00072
   6    2    5.500   1.70475
   7    2    1.420   0.35066
   8    2    4.800   1.56862
   9    2    2.470   0.90422
  10    2    2.140   0.76081
  11    3    2.130   0.75612
  12    3    1.420   0.35066
  13    3    1.840   0.60977
  14    4   14.100   2.64617
  15    4   20.400   3.01553
  16    4    9.460   2.24707
  17    4   24.800   3.21084
  18    5    1.130   0.12222
  20    5    1.080   0.07696
  21    5    1.110   0.10421

MTB >
MTB > onew c3 c1;
SUBC> fisher 0.05;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C3
SOURCE     DF        SS        MS        F        p
C1          4    28.690     7.172    43.16    0.000
ERROR      16     2.659     0.166
TOTAL      20    31.349

                                INDIVIDUAL 95 PCT CI'S FOR MEAN
                                BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -----+---------+---------+---------+-
     1      5    2.7980    0.2669                          (--*---)
     2      5    1.0578    0.5682                 (--*--)
     3      3    0.5722    0.2053           (---*---)
     4      4    2.7799    0.4255                          (--*---)
     5      4   -0.0934    0.3897   (--*---)
                                -----+---------+---------+---------+-
POOLED STDEV =   0.4077              0.0       1.2       2.4       3.6

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.00745
Critical value = 4.33

Intervals for (column level mean) - (row level mean)

              1         2         3         4

     2    0.9507
          2.5296

     3    1.3142   -0.4259
          3.1373    1.3972

     4   -0.8193   -2.5594   -3.1611
          0.8554   -0.8848   -1.2544

     5    2.0541    0.3139   -0.2877    1.9907
          3.7287    1.9886    1.6189    3.7560
Fisher's pairwise comparisons

    Family error rate = 0.259
Individual error rate = 0.0500
```

```
Critical value = 2.120

Intervals for (column level mean) - (row level mean)

               1         2         3         4

     2    1.1936
          2.2868

     3    1.5946   -0.1455
          2.8570    1.1168

     4   -0.5617   -2.3019   -2.8678
          0.5978   -1.1423   -1.5476

     5    2.3116    0.5715    0.0055    2.2622
          3.4712 1.7310    1.3257    3.4845
```

Appendix 1.17: Data of c-AMP for Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{4-13}$ at B-16 Melanoma Cells.

ROW	C1	C2	C3
1	1	199.000	5.29330
2	1	63.000	4.14313
3	1	608.000	6.41017
4	1	74.800	4.31482
5	2	4.290	1.45629
6	2	12.600	2.53370
7	2	20.700	3.03013
8	3	3.500	1.25276
9	3	6.720	1.90509
10	3	1.290	0.25464
11	3	0.507	-0.67924
12	4	8.990	2.19611
13	4	9.620	2.26384
14	4	1.860	0.62058
15	4	4.480	1.49962
16	5	0.720	-0.32850
17	5	1.110	0.10436
18	5	0.606	-0.50088
19	5	1.850	0.61519

```
MTB > onew c3 c1;
SUBC> fisher 0.05;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C3
SOURCE     DF       SS        MS        F        p
C1          4    61.066    15.266    19.56    0.000
ERROR      14    10.926     0.780
TOTAL      18    71.992

                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -----+---------+---------+---------+-
     1      4    5.0404    1.0443                           (----*----)
     2      3    2.3400    0.8046              (-----*-----)
     3      4    0.6833    1.1339       (---*----)
     4      4    1.6450    0.7653           (----*----)
     5      4   -0.0275    0.4984  (----*----)
                                  -----+---------+---------+---------+-
POOLED STDEV =   0.8834             0.0       2.0       4.0       6.0

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.00755

Critical value = 4.41

Intervals for (column level mean) - (row level mean)

               1         2         3         4

     2    0.5963
          4.8044

     3    2.4091   -0.4473
          6.3050    3.7608
```

```
     4    1.4474   -1.4090   -2.9097
          5.3433    2.7990    0.9862

     5    3.1199    0.2635   -1.2372   -0.2755
          7.0158    4.4715    2.6587    3.6205

Fisher's pairwise comparisons

    Family error rate = 0.256
Individual error rate = 0.0500

Critical value = 2.145

Intervals for (column level mean) - (row level mean)

               1         2         3         4

     2    1.2530
          4.1476

     3    3.0171    0.2094
          5.6970    3.1040

     4    2.0554   -0.7523   -2.3017
          4.7353    2.1423    0.3782

     5    3.7279    0.9202   -0.6292    0.3326
          6.4078    3.8148    2.0507    3.0124
```